

Mediators of Hedgehog Signaling Pathways, Compositions and Uses Related Thereto

This application is a continuation-in-part of the U.S. Application to Guicherit et al. entitled "Mediators of Hedgehog Signaling Pathways, Compositions and Uses Related Thereto", filed November 9, 2000, which is a continuation-in-part of U.S. Application No. 09/687,800, filed October 13, 1999, which is a continuation-in-part of U.S. Application No. 09/663,835, filed on September 15, 2000, which is based on U.S. Provisional Applications Nos. 60/154,526, filed September 16, 1999, 60/159,412, filed October 14, 1999, and 60/162,899, filed November 1, 1999, the specifications of all of which are hereby incorporated by reference herein in their entirety. This application further incorporates by reference U.S. Provisional Application Nos. 60/240,564, filed October 13, 2000, and 60/211,919, filed June 16, 2000.

Background of the Invention

Pattern formation is the activity by which embryonic cells form ordered spatial arrangements of differentiated tissues. The physical complexity of higher organisms arises during embryogenesis through the interplay of cell-intrinsic lineage and cell-extrinsic signaling. Inductive interactions are essential to embryonic patterning in vertebrate development from the earliest establishment of the body plan, to the patterning of the organ systems, to the generation of diverse cell types during tissue differentiation (Davidson, E., (1990) Development 108: 365-389; Gurdon, J. B., (1992) Cell 68: 185-199; Jessell, T. M. et al., (1992) Cell 68: 257-270). The effects of developmental cell interactions are varied. Typically, responding cells are diverted from one route of cell differentiation to another by inducing cells that differ from both the uninduced and induced states of the responding cells (inductions). Sometimes cells induce their neighbors to differentiate like themselves (homeogenetic induction); in other cases a cell inhibits its neighbors from differentiating like itself. Cell interactions in early development may be sequential, such that an initial induction between two cell types

leads to a progressive amplification of diversity. Moreover, inductive interactions occur not only in embryos, but in adult cells as well, and can act to establish and maintain morphogenetic patterns as well as induce differentiation (J.B. Gurdon (1992) Cell 68:185-199).

Members of the *Hedgehog* family of signaling molecules mediate many important short- and long-range patterning processes during invertebrate and vertebrate development. In the fly, a single *hedgehog* gene regulates segmental and imaginal disc patterning. In contrast, in vertebrates, a *hedgehog* gene family is involved in the control of left-right asymmetry, polarity in the CNS, somites and limb, organogenesis, chondrogenesis and spermatogenesis.

The first *hedgehog* gene was identified by a genetic screen in the fruitfly *Drosophila melanogaster* (Nüsslein-Volhard, C. and Wieschaus, E. (1980) Nature 287, 795-801). This screen identified a number of mutations affecting embryonic and larval development. In 1992 and 1993, the molecular nature of the *Drosophila hedgehog* (*hh*) gene was reported (C.F., Lee et al. (1992) Cell 71, 33-50), and since then, several *hedgehog* homologues have been isolated from various vertebrate species. While only one *hedgehog* gene has been found in *Drosophila* and other invertebrates, multiple *Hedgehog* genes are present in vertebrates.

The vertebrate family of *hedgehog* genes includes at least four members, e.g., paralogs of the single drosophila *hedgehog* gene. Exemplary hedgehog genes and proteins are described in PCT publications WO 95/18856 and WO 96/17924. Three of these members, herein referred to as Desert *hedgehog* (*Dhh*), Sonic *hedgehog* (*Shh*) and Indian *hedgehog* (*Ihh*), apparently exist in all vertebrates, including fish, birds, and mammals. A fourth member, herein referred to as tiggie-winkle *hedgehog* (*Thh*), appears specific to fish. Desert hedgehog (*Dhh*) is expressed principally in the testes, both in mouse embryonic development and in the adult rodent and human; Indian hedgehog (*Ihh*) is involved in bone development during embryogenesis and in bone formation in the adult; and, *Shh*, which as described above, is primarily involved in morphogenic and neuroinductive activities. Given the critical inductive roles of hedgehog polypeptides in

the development and maintenance of vertebrate organs, the identification of hedgehog interacting proteins is of paramount significance in both clinical and research contexts.

The various *Hedgehog* proteins consist of a signal peptide, a highly conserved N-terminal region, and a more divergent C-terminal domain. In addition to signal sequence cleavage in the secretory pathway (Lee, J.J. *et al.* (1992) Cell 71:33-50; Tabata, T. *et al.* (1992) Genes Dev. 2635-2645; Chang, D.E. *et al.* (1994) Development 120:3339-3353), Hedgehog precursor proteins undergo an internal autoproteolytic cleavage which depends on conserved sequences in the C-terminal portion (Lee *et al.* (1994) Science 266:1528-1537; Porter *et al.* (1995) Nature 374:363-366). This autocleavage leads to a 19 kD N-terminal peptide and a C-terminal peptide of 26-28 kD (Lee *et al.* (1992) *supra*; Tabata *et al.* (1992) *supra*; Chang *et al.* (1994) *supra*; Lee *et al.* (1994) *supra*; Bumcrot, D.A., *et al.* (1995) Mol. Cell. Biol. 15:2294-2303; Porter *et al.* (1995) *supra*; Ekker, S.C. *et al.* (1995) Curr. Biol. 5:944-955; Lai, C.J. *et al.* (1995) Development 121:2349-2360). The N-terminal peptide stays tightly associated with the surface of cells in which it was synthesized, while the C-terminal peptide is freely diffusible both *in vitro* and *in vivo* (Porter *et al.* (1995) Nature 374:363; Lee *et al.* (1994) *supra*; Bumcrot *et al.* (1995) *supra*; Mart', E. *et al.* (1995) Development 121:2537-2547; Roelink, H. *et al.* (1995) Cell 81:445-455). Interestingly, cell surface retention of the N-terminal peptide is dependent on autocleavage, as a truncated form of HH encoded by an RNA which terminates precisely at the normal position of internal cleavage is diffusible *in vitro* (Porter *et al.* (1995) *supra*) and *in vivo* (Porter, J.A. *et al.* (1996) Cell 86, 21-34). Biochemical studies have shown that the autoproteolytic cleavage of the HH precursor protein proceeds through an internal thioester intermediate which subsequently is cleaved in a nucleophilic substitution. It is likely that the nucleophile is a small lipophilic molecule which becomes covalently bound to the C-terminal end of the N-peptide (Porter *et al.* (1996) *supra*), tethering it to the cell surface. The biological implications are profound. As a result of the tethering, a high local concentration of N-terminal Hedgehog peptide is generated on the surface of the Hedgehog producing cells. It is this N-terminal peptide which is both necessary and sufficient for short- and long-range Hedgehog signaling activities in *Drosophila* and vertebrates (Porter *et al.* (1995) *supra*; Ekker *et al.* (1995) *supra*; Lai *et al.* (1995) *supra*; Roelink, H. *et al.* (1995) Cell 81:445-455; Porter *et al.* (1996) *supra*;

Fietz, M.J. *et al.* (1995) Curr. Biol. 5:643-651; Fan, C.-M. *et al.* (1995) Cell 81:457-465; Mart', E., *et al.* (1995) Nature 375:322-325; Lopez-Martinez *et al.* (1995) Curr. Biol 5:791-795; Ekker, S.C. *et al.* (1995) Development 121:2337-2347; Forbes, A.J. *et al.* (1996) Development 122:1125-1135).

HH has been implicated in short- and long-range patterning processes at various sites during *Drosophila* development. In the establishment of segment polarity in early embryos, it has short-range effects which appear to be directly mediated, while in the patterning of the imaginal discs, it induces long range effects via the induction of secondary signals.

In vertebrates, several *hedgehog* genes have been cloned in the past few years. Of these genes, *Shh* has received most of the experimental attention, as it is expressed in different organizing centers which are the sources of signals that pattern neighboring tissues. Recent evidence indicates that *Shh* is involved in these interactions.

The expression of *Shh* starts shortly after the onset of gastrulation in the presumptive midline mesoderm, the node in the mouse (Chang *et al.* (1994) supra; Echelard, Y. *et al.* (1993) Cell 75:1417-1430), the rat (Roelink, H. *et al.* (1994) Cell 76:761-775) and the chick (Riddle, R.D. *et al.* (1993) Cell 75:1401-1416), and the shield in the zebrafish (Ekker *et al.* (1995) supra; Krauss, S. *et al.* (1993) Cell 75:1431-1444). In chick embryos, the *Shh* expression pattern in the node develops a left-right asymmetry, which appears to be responsible for the left-right situs of the heart (Levin, M. *et al.* (1995) Cell 82:803-814).

In the CNS, *Shh* from the notochord and the floorplate appears to induce ventral cell fates. When ectopically expressed, *Shh* leads to a ventralization of large regions of the mid- and hindbrain in mouse (Echelard *et al.* (1993) supra; Goodrich, L.V. *et al.* (1996) Genes Dev. 10:301-312), *Xenopus* (Roelink, H. *et al.* (1994) supra; Ruiz i Altaba, A. *et al.* (1995) Mol. Cell. Neurosci. 6:106-121), and zebrafish (Ekker *et al.* (1995) supra; Krauss *et al.* (1993) supra; Hammerschmidt, M., *et al.* (1996) Genes Dev. 10:647-658). In explants of intermediate neuroectoderm at spinal cord levels, *Shh* protein induces floorplate and motor neuron development with distinct concentration thresholds, floor plate at high and motor neurons at lower concentrations (Roelink *et al.* (1995) supra;

Mart' *et al.* (1995) supra; Tanabe, Y. *et al.* (1995) Curr. Biol. 5:651-658). Moreover, antibody blocking suggests that *Shh* produced by the notochord is required for notochord-mediated induction of motor neuron fates (Mart' *et al.* (1995) supra). Thus, high concentration of *Shh* on the surface of *Shh*-producing midline cells appears to account for the contact-mediated induction of floorplate observed *in vitro* (Placzek, M. *et al.* (1993) Development 117:205-218), and the midline positioning of the floorplate immediately above the notochord *in vivo*. Lower concentrations of *Shh* released from the notochord and the floorplate presumably induce motor neurons at more distant ventrolateral regions in a process that has been shown to be contact-independent *in vitro* (Yamada, T. *et al.* (1993) Cell 73:673-686). In explants taken at midbrain and forebrain levels, *Shh* also induces the appropriate ventrolateral neuronal cell types, dopaminergic (Heynes, M. *et al.* (1995) Neuron 15:35-44; Wang, M.Z. *et al.* (1995) Nature Med. 1:1184-1188) and cholinergic (Ericson, J. *et al.* (1995) Cell 81:747-756) precursors, respectively, indicating that *Shh* is a common inducer of ventral specification over the entire length of the CNS. These observations raise a question as to how the differential response to *Shh* is regulated at particular anteroposterior positions.

Shh from the midline also patterns the paraxial regions of the vertebrate embryo, the somites in the trunk (Fan *et al.* (1995) supra) and the head mesenchyme rostral of the somites (Hammerschmidt *et al.* (1996) supra). In chick and mouse paraxial mesoderm explants, *Shh* promotes the expression of sclerotome specific markers like *Pax1* and *Twist*, at the expense of the dermamyotomal marker *Pax3*. Moreover, filter barrier experiments suggest that *Shh* mediates the induction of the sclerotome directly rather than by activation of a secondary signaling mechanism (Fan, C.-M. and Tessier-Lavigne, M. (1994) Cell 79, 1175-1186).

Shh also induces myotomal gene expression (Hammerschmidt *et al.* (1996) supra; Johnson, R.L. *et al.* (1994) Cell 79:1165-1173; Münsterberg, A.E. *et al.* (1995) Genes Dev. 9:2911-2922; Weinberg, E.S. *et al.* (1996) Development 122:271-280), although recent experiments indicate that members of the WNT family, vertebrate homologues of *Drosophila wingless*, are required in concert (Münsterberg *et al.* (1995) supra). Puzzlingly, myotomal induction in chicks requires higher *Shh* concentrations than the

induction of sclerotomal markers (Münsterberg *et al.* (1995) supra), although the sclerotome originates from somitic cells positioned much closer to the notochord. Similar results were obtained in the zebrafish, where high concentrations of Hedgehog induce myotomal and repress sclerotomal marker gene expression (Hammerschmidt *et al.* (1996) supra). In contrast to amniotes, however, these observations are consistent with the architecture of the fish embryo, as here, the myotome is the predominant and more axial component of the somites. Thus, modulation of *Shh* signaling and the acquisition of new signaling factors may have modified the somite structure during vertebrate evolution.

In the vertebrate limb buds, a subset of posterior mesenchymal cells, the "Zone of polarizing activity" (ZPA), regulates anteroposterior digit identity (reviewed in Honig, L.S. (1981) Nature 291:72-73). Ectopic expression of *Shh* or application of beads soaked in *Shh* peptide mimics the effect of anterior ZPA grafts, generating a mirror image duplication of digits (Chang *et al.* (1994) supra; Lopez-Martinez *et al.* (1995) supra; Riddle *et al.* (1993) supra) (Fig. 2g). Thus, digit identity appears to depend primarily on *Shh* concentration, although it is possible that other signals may relay this information over the substantial distances that appear to be required for AP patterning (100-150 μ m). Similar to the interaction of HH and DPP in the *Drosophila* imaginal discs, *Shh* in the vertebrate limb bud activates the expression of *Bmp2* (Francis, P.H. *et al.* (1994) Development 120:209-218), a *dpp* homologue. However, unlike DPP in *Drosophila*, *Bmp2* fails to mimic the polarizing effect of *Shh* upon ectopic application in the chick limb bud (Francis *et al.* (1994) supra). In addition to anteroposterior patterning, *Shh* also appears to be involved in the regulation of the proximodistal outgrowth of the limbs by inducing the synthesis of the fibroblast growth factor FGF4 in the posterior apical ectodermal ridge (Laufer, E. *et al.* (1994) Cell 79:993-1003; Niswander, L. *et al.* (1994) Nature 371:609-612).

The close relationship between Hedgehog proteins and BMPs is likely to have been conserved at many, but probably not all sites of vertebrate *Hedgehog* expression. For example, in the chick hindgut, *Shh* has been shown to induce the expression of *Bmp4*, another vertebrate *dpp* homologue (Roberts, D.J. *et al.* (1995) Development 121:3163-3174). Furthermore, *Shh* and *Bmp2*, 4, or 6 show a striking correlation in their expression

in epithelial and mesenchymal cells of the stomach, the urogenital system, the lung, the tooth buds and the hair follicles (Bitgood, M.J. and McMahon, A.P. (1995) Dev. Biol. 172:126-138). Further, *Ihh*, one of the two other mouse *Hedgehog* genes, is expressed adjacent to *Bmp* expressing cells in the gut and developing cartilage (Bitgood and McMahon (1995) *supra*).

Recent evidence suggests a model in which *Ihh* plays a crucial role in the regulation of chondrogenic development (Roberts *et al.* (1995) *supra*). During cartilage formation, chondrocytes proceed from a proliferating state via an intermediate, prehypertrophic state to differentiated hypertrophic chondrocytes. *Ihh* is expressed in the prehypertrophic chondrocytes and initiates a signaling cascade that leads to the blockage of chondrocyte differentiation. Its direct target is the perichondrium around the *Ihh* expression domain, which responds by the expression of *Gli* and *Patched* (*Ptc*), conserved transcriptional targets of Hedgehog signals (see below). Most likely, this leads to secondary signaling resulting in the synthesis of parathyroid hormone-related protein (PTHrP) in the periarticular perichondrium. PTHrP itself signals back to the prehypertrophic chondrocytes, blocking their further differentiation. At the same time, PTHrP represses expression of *Ihh*, thereby forming a negative feedback loop that modulates the rate of chondrocyte differentiation.

Patched was originally identified in *Drosophila* as a segment polarity gene, one of a group of developmental genes that affect cell differentiation within the individual segments that occur in a homologous series along the anterior-posterior axis of the embryo. See Hooper, J.E. et al. (1989) Cell 59:751; and Nakano, Y. et al. (1989) Nature 341:508. Patterns of expression of the vertebrate homologue of *patched* suggest its involvement in the development of neural tube, skeleton, limbs, craniofacial structure, and skin.

Genetic and functional studies demonstrate that *patched* is part of the hedgehog signaling cascade, an evolutionarily conserved pathway that regulates expression of a number of downstream genes. See Perrimon, N. (1995) Cell 80:517; and Perrimon, N. (1996) Cell 86:513. *Patched* participates in the constitutive transcriptional repression of the target genes; its effect is opposed by a secreted glycoprotein, encoded by hedgehog,

or a vertebrate homologue, which induces transcriptional activation. Genes under control of this pathway include members of the Wnt and TGF-beta families.

Patched proteins possess two large extracellular domains, twelve transmembrane segments, and several cytoplasmic segments. See Hooper, supra; Nakano, supra; Johnson, R.L. et al. (1996) Science 272:1668; and Hahn, H. et al. (1996) Cell 85:841. The biochemical role of *patched* in the hedgehog signaling pathway is unclear. Direct interaction with the hedgehog protein has, however, been reported (Chen, Y. et al. (1996) Cell 87:553), and *patched* may participate in a hedgehog receptor complex along with another transmembrane protein encoded by the *smoothed* gene. See Perrimon, supra; and Chen, supra.

The human homologue of *patched* was recently cloned and mapped to chromosome 9q22.3. See Johnson, supra; and Hahn, supra. This region has been implicated in basal cell nevus syndrome (BCNS), which is characterized by developmental abnormalities including rib and craniofacial alterations, abnormalities of the hands and feet, and spina bifida.

Sporadic tumors also demonstrated a loss of both functional alleles of *patched*. Of twelve tumors in which *patched* mutations were identified with a single strand conformational polymorphism screening assay, nine had chromosomal deletion of the second allele and the other three had inactivating mutations in both alleles (Gailani, supra). The alterations did not occur in the corresponding germline DNA.

Most of the identified mutations resulted in premature stop codons or frame shifts. Lench, N.J., et al., *Hum. Genet.* 1997 Oct; 100(5-6): 497-502. Several, however, were point mutations leading to amino acid substitutions in either extracellular or cytoplasmic domains. These sites of mutation may indicate functional importance for interaction with extracellular proteins or with cytoplasmic members of the downstream signaling pathway.

The involvement of *patched* in the inhibition of gene expression and the occurrence of frequent allelic deletions of *patched* in BCC support a tumor suppressor function for this gene. Its role in the regulation of gene families known to be involved in

cell signaling and intercellular communication provides a possible mechanism of tumor suppression.

Summary of the Invention

The present invention makes available methods and reagents for inhibiting activation of the hedgehog signaling pathway, both in normal cells and cells having an abnormal phenotype such as hedgehog gain-of-function, by contacting the cell with an agent, such as a small molecule, in a sufficient amount to antagonize a normal hedgehog pathway. In certain embodiments of the present invention, a *hedgehog* pathway antagonist, according to the present invention, inhibits *hedgehog*-dependent transcriptional activation, such as expression of a *gli* gene (particularly *Gli-1*) but does not inhibit *ptc*^{loss-of-function}-dependent transcriptional activation. In certain preferred embodiments, the antagonist inhibits expression of a *gli* gene by at least 5%, at least 10%, at least 20%, or even at least about 50% relative to a control in the absence of the antagonist. The coding sequences for exemplary human *Gli* genes include, for example, the *Gli-1* gene sequence of GenBank accession X07384 and the *Gli-2* gene sequence of GenBank accession AB007298. See also Kinzler et al. *Nature* 1988, 332, 371. The level of *gli* expression can be determined, for example, by measuring the level of mRNA (transcription) or the level of protein (translation). While not wishing to be bound by any particular theory, the antagonist may exert such inhibitory activity by binding to *patched* or a complex including *patched*.

In certain embodiments, a compound as set forth above may be a small organic molecule, e.g., may have a molecular weight of less than about 2000 amu, less than about 1500 amu, or even less than about 1000 amu. In certain preferred embodiments, the antagonist is a non-peptidyl organic molecule. In certain embodiments, a compound useful in the present invention, such as described above, may have an IC₅₀ for inhibiting one or more *hedgehog* activities (such as reduction of *gli* expression) of less than about 1000 nM, less than about 100 nM, less than about 10 nM, or even less than about 1 nM.

Brief Description of the Drawings

Figures 1-31 depict reactions useful for synthesizing compounds according to the present invention.

Figures 32a-m illustrate representative compounds according to the present invention.

Figure 33 presents biological testing results of an exemplary compound of the invention.

Figure 34 presents the effects of an exemplary compound of the invention on lung tissue.

Detailed Description of the Invention

I. Overview

The present invention relates to the discovery that signal transduction pathways regulated by *hedgehog*, *patched* (*ptc*), *gli* and/or *smoothened* can be inhibited, at least in part, by small molecules. While not wishing to be bound by any particular theory, the activation of a receptor may be the mechanism by which these agents act. For example, the subject compounds may interact with or bind to *patched*.

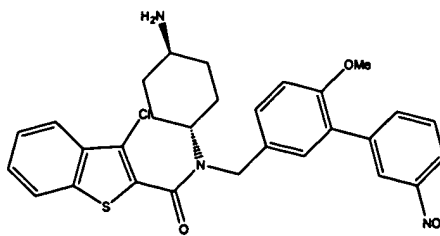
It is, therefore, specifically contemplated that these small molecules which interfere with aspects of *hedgehog*, *ptc*, or *smoothened* signal transduction activity will likewise be capable of inhibiting proliferation (or other biological consequences) in cells having a *hedgehog* gain-of-function phenotype. Similarly, the subject compounds may be useful for treating conditions wherein inhibition of *hedgehog* signalling is desirable. In preferred embodiments, the subject inhibitors are organic molecules having a molecular weight less than 2500 amu, more preferably less than 1500 amu, and even more preferably less than 750 amu, and are capable of inhibiting at least some of the biological activities of hedgehog proteins, preferably specifically in target cells.

Thus, the methods of the present invention include the use of small molecules which agonize *ptc* inhibition of *hedgehog* signalling in the regulation of repair and/or functional performance of a wide range of cells, tissues and organs, including normal tissues and those having the phenotype of *hedgehog* gain-of-function. For instance, the subject method has therapeutic and cosmetic applications ranging from regulation of

neural tissues, bone and cartilage formation and repair, regulation of spermatogenesis, regulation of smooth muscle, regulation of lung, liver and tissue of other organs arising from the primitive gut, regulation of hematopoietic function, regulation of skin and hair growth, etc. Moreover, the subject methods can be performed on cells which are provided in culture (*in vitro*), or on cells in a whole animal (*in vivo*). See, for example, PCT publications WO 95/18856 and WO 96/17924 (the specifications of which are expressly incorporated by reference herein).

In certain embodiments, *hedgehog* antagonists useful in the present inhibit *hedgehog*-dependent transcriptional regulation, such as expression of the *gli1* or *ptc* genes. Such antagonists can thus inhibit the *hedgehog*-dependent pathway activation resulting from, for example, increased levels of *hedgehog* protein, but do not inhibit the upregulation of the *hedgehog* pathway observed as a result of *patched*^{loss-of-function} or *smoothened*^{gain-of-function} mutations, or other activation of the pathway at a point downstream of *patched*, such as by elevated levels of cAMP and the like.

In certain embodiments, *hedgehog* antagonists useful in the present invention bind to *patched*. Such antagonists can thus inhibit the *hedgehog*-pathway agonizing effect of, for example, increased levels of *hedgehog* protein, but do not inhibit the upregulation of the *hedgehog* pathway observed in *patched*-null cells, or activation of the pathway at a point downstream of *patched*, such as by cAMP. Inhibition of the *hedgehog* pathway by a *hedgehog* antagonist may be quantified, for example, by detecting the decrease in *gli* transcription in the presence of the antagonist relative to a control in the absence of antagonist. For example, a decrease of at least 5%, at least 10%, at least 20%, or even at least 50% may be indicative of *hedgehog* pathway inhibition by a test compound. Similarly, compounds of the present invention may mimic or recapitulate the activity of an antibody which binds a *hedgehog* protein, but do not block the *hedgehog*-agonizing



activity of the following agonist:

, e.g., do not induce a

decrease of more than 50%, more than 20%, more than 10%, or even more than 5% of the *gli-1* upregulation induced by the agonist in the absence of the *hedgehog* antagonist.

In a preferred embodiment, the subject method can be to treat epithelial cells having a phenotype of *hedgehog* gain-of-function. In another preferred embodiment, the subject method can be used as part of a treatment regimen for malignant medulloblastoma and other primary CNS malignant neuroectodermal tumors. In yet another embodiment, the subject compounds may be used to treat basal cell carcinoma.

In another aspect, the present invention provides pharmaceutical preparations comprising, as an active ingredient, a *hedgehog* antagonist or *ptc* agonist such as described herein, formulated in an amount sufficient to inhibit, *in vivo*, proliferation or other biological consequences of *hedgehog* gain-of-function.

The subject treatments using *hedgehog* antagonists can be effective for both human and animal subjects. Animal subjects to which the invention is applicable extend to both domestic animals and livestock, raised either as pets or for commercial purposes. Examples are dogs, cats, cattle, horses, sheep, hogs, and goats.

II. Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

The phrase "aberrant modification or mutation" of a gene refers to such genetic lesions as, for example, deletions, substitution or addition of nucleotides to a gene, as well as gross chromosomal rearrangements of the gene and/or abnormal methylation of the gene. Likewise, mis-expression of a gene refers to aberrant levels of transcription of the gene relative to those levels in a normal cell under similar conditions, as well as non-wild-type splicing of mRNA transcribed from the gene.

"Basal cell carcinomas" exist in a variety of clinical and histological forms such as nodular-ulcerative, superficial, pigmented, morphealike, fibroepithelioma and nevoid syndrome. Basal cell carcinomas are the most common cutaneous neoplasms found in humans. The majority of new cases of nonmelanoma skin cancers fall into this category.

"Burn wounds" refer to cases where large surface areas of skin have been removed or lost from an individual due to heat and/or chemical agents.

The term "carcinoma" refers to a malignant new growth made up of epithelial cells tending to infiltrate surrounding tissues and to give rise to metastases. Exemplary carcinomas include: "basal cell carcinoma", which is an epithelial tumor of the skin that, while seldom metastasizing, has potentialities for local invasion and destruction; "squamous cell carcinoma", which refers to carcinomas arising from squamous epithelium and having cuboid cells; "carcinosarcoma", which include malignant tumors composed of carcinomatous and sarcomatous tissues; "adenocystic carcinoma", carcinoma marked by cylinders or bands of hyaline or mucinous stroma separated or surrounded by nests or cords of small epithelial cells, occurring in the mammary and salivary glands, and mucous glands of the respiratory tract; "epidermoid carcinoma", which refers to cancerous cells which tend to differentiate in the same way as those of the epidermis; i.e., they tend to form prickly cells and undergo cornification; "nasopharyngeal carcinoma", which refers to a malignant tumor arising in the epithelial lining of the space behind the nose; and "renal cell carcinoma", which pertains to carcinoma of the renal parenchyma composed of tubular cells in varying arrangements. Other carcinomatous epithelial growths are "papillomas", which refers to benign tumors derived from epithelium and having a papillomavirus as a causative agent; and "epidermoidomas", which refers to a cerebral or meningeal tumor formed by inclusion of ectodermal elements at the time of closure of the neural groove.

The "corium" or "dermis" refers to the layer of the skin deep to the epidermis, consisting of a dense bed of vascular connective tissue, and containing the nerves and terminal organs of sensation. The hair roots, and sebaceous and sweat glands are structures of the epidermis which are deeply embedded in the dermis.

"Dental tissue" refers to tissue in the mouth which is similar to epithelial tissue, for example gum tissue. The method of the present invention is useful for treating periodontal disease.

"Dermal skin ulcers" refer to lesions on the skin caused by superficial loss of tissue, usually with inflammation. Dermal skin ulcers which can be treated by the method of the present invention include decubitus ulcers, diabetic ulcers, venous stasis ulcers and arterial ulcers. Decubitus wounds refer to chronic ulcers that result from pressure applied to areas of the skin for extended periods of time. Wounds of this type are often called bedsores or pressure sores. Venous stasis ulcers result from the stagnation of blood or other fluids from defective veins. Arterial ulcers refer to necrotic skin in the area around arteries having poor blood flow.

The term "ED₅₀" means the dose of a drug which produces 50% of its maximum response or effect.

An "effective amount" of, e.g., a *hedgehog* antagonist, with respect to the subject method of treatment, refers to an amount of the antagonist in a preparation which, when applied as part of a desired dosage regimen brings about, e.g., a change in the rate of cell proliferation and/or the state of differentiation of a cell and/or rate of survival of a cell according to clinically acceptable standards for the disorder to be treated or the cosmetic purpose.

The terms "epithelia", "epithelial" and "epithelium" refer to the cellular covering of internal and external body surfaces (cutaneous, mucous and serous), including the glands and other structures derived therefrom, e.g., corneal, esophageal, epidermal, and hair follicle epithelial cells. Other exemplary epithelial tissue includes: olfactory epithelium, which is the pseudostratified epithelium lining the olfactory region of the nasal cavity, and containing the receptors for the sense of smell; glandular epithelium, which refers to epithelium composed of secreting cells; squamous epithelium, which refers to epithelium composed of flattened plate-like cells. The term epithelium can also refer to transitional epithelium, like that which is characteristically found lining hollow organs that are subject to great mechanical change due to contraction and distention, e.g.,

tissue which represents a transition between stratified squamous and columnar epithelium.

The term "epithelialization" refers to healing by the growth of epithelial tissue over a denuded surface.

The term "epidermal gland" refers to an aggregation of cells associated with the epidermis and specialized to secrete or excrete materials not related to their ordinary metabolic needs. For example, "sebaceous glands" are holocrine glands in the corium that secrete an oily substance and sebum. The term "sweat glands" refers to glands that secrete sweat, situated in the corium or subcutaneous tissue, opening by a duct on the body surface.

The term "epidermis" refers to the outermost and nonvascular layer of the skin, derived from the embryonic ectoderm, varying in thickness from 0.07-1.4 mm. On the palmar and plantar surfaces it comprises, from within outward, five layers: basal layer composed of columnar cells arranged perpendicularly; prickle-cell or spinous layer composed of flattened polyhedral cells with short processes or spines; granular layer composed of flattened granular cells; clear layer composed of several layers of clear, transparent cells in which the nuclei are indistinct or absent; and horny layer composed of flattened, cornified non-nucleated cells. In the epidermis of the general body surface, the clear layer is usually absent.

"Excisional wounds" include tears, abrasions, cuts, punctures or lacerations in the epithelial layer of the skin and may extend into the dermal layer and even into subcutaneous fat and beyond. Excisional wounds can result from surgical procedures or from accidental penetration of the skin.

The "growth state" of a cell refers to the rate of proliferation of the cell and/or the state of differentiation of the cell. An "altered growth state" is a growth state characterized by an abnormal rate of proliferation, e.g., a cell exhibiting an increased or decreased rate of proliferation relative to a normal cell.

The term "hair" refers to a threadlike structure, especially the specialized epidermal structure composed of keratin and developing from a papilla sunk in the

corium, produced only by mammals and characteristic of that group of animals. Also, "hair" may refer to the aggregate of such hairs. A "hair follicle" refers to one of the tubular-invasions of the epidermis enclosing the hairs, and from which the hairs grow. "Hair follicle epithelial cells" refers to epithelial cells which surround the dermal papilla in the hair follicle, e.g., stem cells, outer root sheath cells, matrix cells, and inner root sheath cells. Such cells may be normal non-malignant cells, or transformed/immortalized cells.

The term "*hedgehog* antagonist" refers to an agent which potentiates or recapitulates the bioactivity of *patched*, such as to repress transcription of target genes. Preferred *hedgehog* antagonists can be used to overcome a *ptc* loss-of-function and/or a *smoothed* gain-of-function, the latter also being referred to as *smoothed* antagonists. The term '*hedgehog* antagonist' as used herein refers not only to any agent that may act by directly inhibiting the normal function of the *hedgehog* protein, but also to any agent that inhibits the *hedgehog* signalling pathway, and thus recapitulates the function of *ptc*.

The term "*hedgehog* gain-of-function" refers to an aberrant modification or mutation of a *ptc* gene, *hedgehog* gene, or *smoothed* gene, or a decrease (or loss) in the level of expression of such a gene, which results in a phenotype which resembles contacting a cell with a *hedgehog* protein, e.g., aberrant activation of a *hedgehog* pathway. The gain-of-function may include a loss of the ability of the *ptc* gene product to regulate the level of expression of *Ci* genes, e.g., *Gli1*, *Gli2*, and *Gli3*. The term '*hedgehog* gain-of-function' is also used herein to refer to any similar cellular phenotype (e.g., exhibiting excess proliferation) which occurs due to an alteration anywhere in the *hedgehog* signal transduction pathway, including, but not limited to, a modification or mutation of *hedgehog* itself. For example, a tumor cell with an abnormally high proliferation rate due to activation of the *hedgehog* signalling pathway would have a '*hedgehog* gain-of-function' phenotype, even if *hedgehog* is not mutated in that cell.

As used herein, "immortalized cells" refers to cells which have been altered via chemical and/or recombinant means such that the cells have the ability to grow through an indefinite number of divisions in culture.

"Internal epithelial tissue" refers to tissue inside the body which has characteristics similar to the epidermal layer in the skin. Examples include the lining of the intestine. The method of the present invention is useful for promoting the healing of certain internal wounds, for example wounds resulting from surgery.

The term "keratosis" refers to proliferative skin disorder characterized by hyperplasia of the horny layer of the epidermis. Exemplary keratotic disorders include keratosis follicularis, keratosis palmaris et plantaris, keratosis pharyngea, keratosis pilaris, and actinic keratosis.

The term "LD₅₀" means the dose of a drug which is lethal in 50% of test subjects.

The term "nail" refers to the horny cutaneous plate on the dorsal surface of the distal end of a finger or toe.

The term "*patched* loss-of-function" refers to an aberrant modification or mutation of a *ptc* gene, or a decreased level of expression of the gene, which results in a phenotype which resembles contacting a cell with a hedgehog protein, e.g., aberrant activation of a hedgehog pathway. The loss-of-function may include a loss of the ability of the *ptc* gene product to regulate the level of expression of Ci genes, e.g., *Gli1*, *Gli2* and *Gli3*.

A "patient" or "subject" to be treated by the subject method can mean either a human or non-human animal.

The term "prodrug" is intended to encompass compounds which, under physiological conditions, are converted into the therapeutically active agents of the present invention. A common method for making a prodrug is to include selected moieties which are hydrolyzed under physiological conditions to reveal the desired molecule. In other embodiments, the prodrug is converted by an enzymatic activity of the host animal.

As used herein, "proliferating" and "proliferation" refer to cells undergoing mitosis.

Throughout this application, the term "proliferative skin disorder" refers to any disease/disorder of the skin marked by unwanted or aberrant proliferation of cutaneous

tissue. These conditions are typically characterized by epidermal cell proliferation or incomplete cell differentiation, and include, for example, X-linked ichthyosis, psoriasis, atopic dermatitis, allergic contact dermatitis, epidermolytic hyperkeratosis, and seborrheic dermatitis. For example, epidermodysplasia is a form of faulty development of the epidermis. Another example is "epidermolysis", which refers to a loosened state of the epidermis with formation of blebs and bullae either spontaneously or at the site of trauma.

As used herein, the term "psoriasis" refers to a hyperproliferative skin disorder which alters the skin's regulatory mechanisms. In particular, lesions are formed which involve primary and secondary alterations in epidermal proliferation, inflammatory responses of the skin, and an expression of regulatory molecules such as lymphokines and inflammatory factors. Psoriatic skin is morphologically characterized by an increased turnover of epidermal cells, thickened epidermis, abnormal keratinization, inflammatory cell infiltrates into the dermis layer and polymorphonuclear leukocyte infiltration into the epidermis layer resulting in an increase in the basal cell cycle. Additionally, hyperkeratotic and parakeratotic cells are present.

The term "skin" refers to the outer protective covering of the body, consisting of the corium and the epidermis, and is understood to include sweat and sebaceous glands, as well as hair follicle structures. Throughout the present application, the adjective "cutaneous" may be used, and should be understood to refer generally to attributes of the skin, as appropriate to the context in which they are used.

The term "*smoothened* gain-of-function" refers to an aberrant modification or mutation of a *smo* gene, or an increased level of expression of the gene, which results in a phenotype which resembles contacting a cell with a hedgehog protein, e.g., aberrant activation of a hedgehog pathway. While not wishing to be bound by any particular theory, it is noted that *ptc* may not signal directly into the cell, but rather interact with *smoothened*, another membrane-bound protein located downstream of *ptc* in *hedgehog* signaling (Marigo et al., (1996) Nature 384: 177-179). The gene *smo* is a segment-polarity gene required for the correct patterning of every segment in *Drosophila* (Alcedo et al., (1996) Cell 86: 221-232). Human homologs of *smo* have been identified. See, for

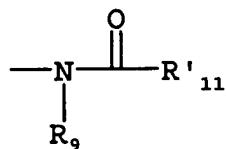
example, Stone et al. (1996) Nature 384:129-134, and GenBank accession U84401. The *smoothened* gene encodes an integral membrane protein with characteristics of heterotrimeric G-protein-coupled receptors; i.e., 7-transmembrane regions. This protein shows homology to the *Drosophila* *Frizzled* (Fz) protein, a member of the *wingless* pathway. It was originally thought that *smo* encodes a receptor of the Hh signal. However, this suggestion was subsequently disproved, as evidence for *ptc* being the Hh receptor was obtained. Cells that express *Smo* fail to bind Hh, indicating that *smo* does not interact directly with Hh (Nusse, (1996) Nature 384: 119-120). Rather, the binding of *Sonic hedgehog* (SHH) to its receptor, *PTCH*, is thought to prevent normal inhibition by *PTCH* of *smoothened* (SMO), a seven-span transmembrane protein.

Recently, it has been reported that activating *smoothened* mutations occur in sporadic basal cell carcinoma, Xie et al. (1998) Nature 391: 90-2, and primitive neuroectodermal tumors of the central nervous system, Reifenberger et al. (1998) Cancer Res 58: 1798-803.

The term "therapeutic index" refers to the therapeutic index of a drug defined as LD_{50}/ED_{50} .

As used herein, "transformed cells" refers to cells which have spontaneously converted to a state of unrestrained growth, i.e., they have acquired the ability to grow through an indefinite number of divisions in culture. Transformed cells may be characterized by such terms as neoplastic, anaplastic and/or hyperplastic, with respect to their loss of growth control.

The term "acylamino" is art-recognized and refers to a moiety that can be represented by the general formula:



wherein R_9 is as defined above, and R'_{11} represents a hydrogen, an alkyl, an alkenyl or $-(CH_2)_m-R_8$, where m and R_8 are as defined above.

Herein, the term "aliphatic group" refers to a straight-chain, branched-chain, or cyclic aliphatic hydrocarbon group and includes saturated and unsaturated aliphatic groups, such as an alkyl group, an alkenyl group, and an alkynyl group.

The terms "alkenyl" and "alkynyl" refer to unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double or triple bond respectively.

The terms "alkoxyl" or "alkoxy" as used herein refers to an alkyl group, as defined above, having an oxygen radical attached thereto. Representative alkoxyl groups include methoxy, ethoxy, propyloxy, tert-butoxy and the like. An "ether" is two hydrocarbons covalently linked by an oxygen. Accordingly, the substituent of an alkyl that renders that alkyl an ether is or resembles an alkoxyl, such as can be represented by one of -O-alkyl, -O-alkenyl, -O-alkynyl, -O-(CH₂)_m-R₈, where m and R_8 are described above.

The term "alkyl" refers to the radical of saturated aliphatic groups, including straight-chain alkyl groups, branched-chain alkyl groups, cycloalkyl (alicyclic) groups, alkyl-substituted cycloalkyl groups, and cycloalkyl-substituted alkyl groups. In preferred embodiments, a straight chain or branched chain alkyl has 30 or fewer carbon atoms in its backbone (e.g., C₁-C₃₀ for straight chains, C₃-C₃₀ for branched chains), and more preferably 20 or fewer. Likewise, preferred cycloalkyls have from 3-10 carbon atoms in their ring structure, and more preferably have 5, 6 or 7 carbons in the ring structure.

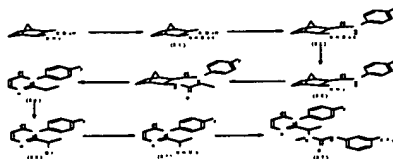
Moreover, the term "alkyl" (or "lower alkyl") as used throughout the specification, examples, and claims is intended to include both "unsubstituted alkyls" and "substituted alkyls", the latter of which refers to alkyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, a halogen, a hydroxyl, a carbonyl (such as a carboxyl, an alkoxycarbonyl, a formyl, or an acyl), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an alkoxyl, a phosphoryl, a phosphate, a phosphonate, a phosphinate, an amino, an amido, an amidine, an imine, a cyano, a nitro, an azido, a sulfhydryl, an

alkylthio, a sulfate, a sulfonate, a sulfamoyl, a sulfonamido, a sulfonyl, a heterocyclyl, an aralkyl, or an aromatic or heteroaromatic moiety. It will be understood by those skilled in the art that the moieties substituted on the hydrocarbon chain can themselves be substituted, if appropriate. For instance, the substituents of a substituted alkyl may include substituted and unsubstituted forms of amino, azido, imino, amido, phosphoryl (including phosphonate and phosphinate), sulfonyl (including sulfate, sulfonamido, sulfamoyl and sulfonate), and silyl groups, as well as ethers, alkylthios, carbonyls (including ketones, aldehydes, carboxylates, and esters), $-\text{CF}_3$, $-\text{CN}$ and the like. Exemplary substituted alkyls are described below. Cycloalkyls can be further substituted with alkyls, alkenyls, alkoxys, alkylthios, aminoalkyls, carbonyl-substituted alkyls, $-\text{CF}_3$, $-\text{CN}$, and the like.

Unless the number of carbons is otherwise specified, "lower alkyl" as used herein means an alkyl group, as defined above, but having from one to ten carbons, more preferably from one to six carbon atoms in its backbone structure. Likewise, "lower alkenyl" and "lower alkynyl" have similar chain lengths. Throughout the application, preferred alkyl groups are lower alkyls. In preferred embodiments, a substituent designated herein as alkyl is a lower alkyl.

The term "alkylthio" refers to an alkyl group, as defined above, having a sulfur radical attached thereto. In preferred embodiments, the "alkylthio" moiety is represented by one of $-\text{S-alkyl}$, $-\text{S-alkenyl}$, $-\text{S-alkynyl}$, and $-\text{S-(CH}_2)_m\text{-R}_8$, wherein m and R_8 are defined above. Representative alkylthio groups include methylthio, ethylthio, and the like.

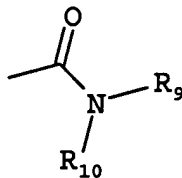
The terms "amine" and "amino" are art-recognized and refer to both unsubstituted and substituted amines, e.g., a moiety that can be represented by the general formula:



wherein R_9 , R_{10} and R'_{10} each independently represent a hydrogen, an alkyl, an alkenyl, $-(\text{CH}_2)_m\text{-R}_8$, or R_9 and R_{10} taken together with the N atom to which they are attached

complete a heterocycle having from 4 to 8 atoms in the ring structure; R₈ represents an aryl, a cycloalkyl, a cycloalkenyl, a heterocycle or a polycycle; and m is zero or an integer in the range of 1 to 8. In preferred embodiments, only one of R₉ or R₁₀ can be a carbonyl, e.g., R₉, R₁₀ and the nitrogen together do not form an imide. In even more preferred embodiments, R₉ and R₁₀ (and optionally R'₁₀) each independently represent a hydrogen, an alkyl, an alkenyl, or -(CH₂)_m-R₈. Thus, the term "alkylamine" as used herein means an amine group, as defined above, having a substituted or unsubstituted alkyl attached thereto, i.e., at least one of R₉ and R₁₀ is an alkyl group.

The term "amido" is art-recognized as an amino-substituted carbonyl and includes a moiety that can be represented by the general formula:



wherein R₉, R₁₀ are as defined above. Preferred embodiments of the amide will not include imides which may be unstable.

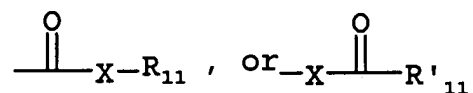
The term "aralkyl", as used herein, refers to an alkyl group substituted with an aryl group (e.g., an aromatic or heteroaromatic group).

The term "aryl" as used herein includes 5-, 6-, and 7-membered single-ring aromatic groups that may include from zero to four heteroatoms, for example, benzene, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, triazole, pyrazole, pyridine, pyrazine, pyridazine and pyrimidine, and the like. Those aryl groups having heteroatoms in the ring structure may also be referred to as "aryl heterocycles" or "heteroaromatics." The aromatic ring can be substituted at one or more ring positions with such substituents as described above, for example, halogen, azide, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, alkoxyl, amino, nitro, sulfhydryl, imino, amido, phosphate, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, sulfonamido, ketone, aldehyde, ester, heterocyclyl, aromatic or heteroaromatic moieties, -CF₃, -CN, or the like. The term "aryl" also includes polycyclic ring systems having two

or more cyclic rings in which two or more carbons are common to two adjoining rings (the rings are "fused rings") wherein at least one of the rings is aromatic, e.g., the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls and/or heterocyclyls.

The term "carbocycle", as used herein, refers to an aromatic or non-aromatic ring in which each atom of the ring is carbon.

The term "carbonyl" is art-recognized and includes such moieties as can be represented by the general formula:



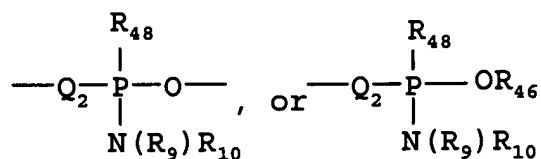
wherein X is a bond or represents an oxygen or a sulfur, and R₁₁ represents a hydrogen, an alkyl, an alkenyl, -(CH₂)_m-R₈ or a pharmaceutically acceptable salt, R'₁₁ represents a hydrogen, an alkyl, an alkenyl or -(CH₂)_m-R₈, where m and R₈ are as defined above. Where X is an oxygen and R₁₁ or R'₁₁ is not hydrogen, the formula represents an "ester". Where X is an oxygen, and R₁₁ is as defined above, the moiety is referred to herein as a carboxyl group, and particularly when R₁₁ is a hydrogen, the formula represents a "carboxylic acid". Where X is an oxygen, and R'₁₁ is hydrogen, the formula represents a "formate". In general, where the oxygen atom of the above formula is replaced by sulfur, the formula represents a "thiocarbonyl" group. Where X is a sulfur and R₁₁ or R'₁₁ is not hydrogen, the formula represents a "thioester." Where X is a sulfur and R₁₁ is hydrogen, the formula represents a "thiocarboxylic acid." Where X is a sulfur and R'₁₁ is hydrogen, the formula represents a "thiolformate." On the other hand, where X is a bond, and R₁₁ is not hydrogen, the above formula represents a "ketone" group. Where X is a bond, and R₁₁ is hydrogen, the above formula represents an "aldehyde" group.

The term "heteroatom" as used herein means an atom of any element other than carbon or hydrogen. Preferred heteroatoms are boron, nitrogen, oxygen, phosphorus, sulfur and selenium.

The terms "heterocyclyl" or "heterocyclic group" refer to 3- to 10-membered ring structures, more preferably 3- to 7-membered rings, whose ring structures include one to four heteroatoms. Heterocycles can also be polycycles. Heterocyclyl groups include, for example, thiophene, thianthrene, furan, pyran, isobenzofuran, chromene, xanthene, phenoxathiin, pyrrole, imidazole, pyrazole, isothiazole, isoxazole, pyridine, pyrazine, pyrimidine, pyridazine, indolizine, isoindole, indole, indazole, purine, quinolizine, isoquinoline, quinoline, phthalazine, naphthyridine, quinoxaline, quinazoline, cinnoline, pteridine, carbazole, carboline, phenanthridine, acridine, pyrimidine, phenanthroline, phenazine, phenarsazine, phenothiazine, furazan, phenoxazine, pyrrolidine, oxolane, thiolane, oxazole, piperidine, piperazine, morpholine, lactones, lactams such as azetidinones and pyrrolidinones, sultams, sultones, and the like. The heterocyclic ring can be substituted at one or more positions with such substituents as described above, as for example, halogen, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, amino, nitro, sulfhydryl, imino, amido, phosphate, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, ketone, aldehyde, ester, a heterocyclyl, an aromatic or heteroaromatic moiety, -CF₃, -CN, or the like.

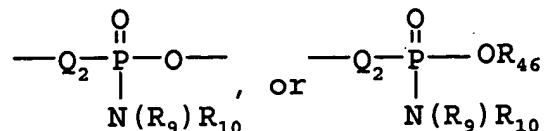
As used herein, the term "nitro" means -NO₂; the term "halogen" designates -F, -Cl, -Br or -I; the term "sulfhydryl" means -SH; the term "hydroxyl" means -OH; and the term "sulfonyl" means -SO₂-.

A "phosphonamidite" can be represented in the general formula:



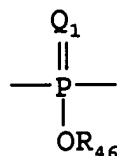
wherein R₉ and R₁₀ are as defined above, Q₂ represents O, S or N, and R₄₈ represents a lower alkyl or an aryl, Q₂ represents O, S or N.

A "phosphoramidite" can be represented in the general formula:

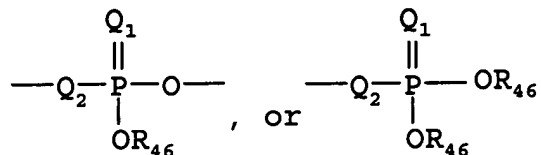


wherein R_9 and R_{10} are as defined above, and Q_2 represents O, S or N.

A "phosphoryl" can in general be represented by the formula:



wherein Q_1 represented S or O, and R_{46} represents hydrogen, a lower alkyl or an aryl. When used to substitute, for example, an alkyl, the phosphoryl group of the phosphorylalkyl can be represented by the general formula:



wherein Q_1 represented S or O, and each R_{46} independently represents hydrogen, a lower alkyl or an aryl, Q_2 represents O, S or N. When Q_1 is an S, the phosphoryl moiety is a "phosphorothioate".

The terms "polycyclyl" or "polycyclic group" refer to two or more rings (e.g., cycloalkyls, cycloalkenyls, cycloalkynyls, aryls and/or heterocyclyls) in which two or more carbons are common to two adjoining rings, e.g., the rings are "fused rings". Rings that are joined through non-adjacent atoms are termed "bridged" rings. Each of the rings of the polycycle can be substituted with such substituents as described above, as for example, halogen, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, amino, nitro, sulfhydryl, imino, amido, phosphate, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, ketone, aldehyde, ester, a heterocyclyl, an aromatic or heteroaromatic moiety, $-\text{CF}_3$, $-\text{CN}$, or the like.

The phrase "protecting group" as used herein means temporary substituents which protect a potentially reactive functional group from undesired chemical transformations.

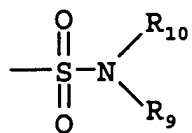
Examples of such protecting groups include esters of carboxylic acids, silyl ethers of alcohols, and acetals and ketals of aldehydes and ketones, respectively. The field of protecting group chemistry has been reviewed (Greene, T.W.; Wuts, P.G.M. *Protective Groups in Organic Synthesis*, 2nd ed.; Wiley: New York, 1991).

A "selenoalkyl" refers to an alkyl group having a substituted seleno group attached thereto. Exemplary "selenoethers" which may be substituted on the alkyl are selected from one of -Se-alkyl, -Se-alkenyl, -Se-alkynyl, and -Se-(CH₂)_m-R₈, m and R₈ being defined above.

As used herein, the term "substituted" is contemplated to include all permissible substituents of organic compounds. In a broad aspect, the permissible substituents include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and nonaromatic substituents of organic compounds. Illustrative substituents include, for example, those described herein above. The permissible substituents can be one or more and the same or different for appropriate organic compounds. For purposes of this invention, the heteroatoms such as nitrogen may have hydrogen substituents and/or any permissible substituents of organic compounds described herein which satisfy the valences of the heteroatoms. This invention is not intended to be limited in any manner by the permissible substituents of organic compounds.

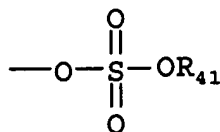
It will be understood that "substitution" or "substituted with" includes the implicit proviso that such substitution is in accordance with permitted valence of the substituted atom and the substituent, and that the substitution results in a stable compound, e.g., which does not spontaneously undergo transformation such as by rearrangement, cyclization, elimination, etc.

The term "sulfamoyl" is art-recognized and includes a moiety that can be represented by the general formula:



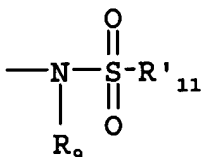
in which R₉ and R₁₀ are as defined above.

The term "sulfate" is art recognized and includes a moiety that can be represented by the general formula:



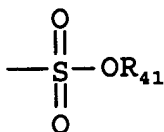
in which R₄₁ is as defined above.

The term "sulfonamido" is art recognized and includes a moiety that can be represented by the general formula:



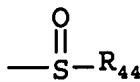
in which R₉ and R'₁₁ are as defined above.

The term "sulfonate" is art-recognized and includes a moiety that can be represented by the general formula:



in which R₄₁ is an electron pair, hydrogen, alkyl, cycloalkyl, or aryl.

The terms "sulfoxido" or "sulfinyl", as used herein, refers to a moiety that can be represented by the general formula:



in which R₄₄ is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aralkyl, or aryl.

Analogous substitutions can be made to alkenyl and alkynyl groups to produce, for example, aminoalkenyls, aminoalkynyls, amidoalkenyls, amidoalkynyls,

iminoalkenyls, iminoalkynyls, thioalkenyls, thioalkynyls, carbonyl-substituted alkenyls or alkynyls.

As used herein, the definition of each expression, e.g., alkyl, m, n, etc., when it occurs more than once in any structure, is intended to be independent of its definition elsewhere in the same structure.

The terms triflyl, tosyl, mesyl, and nonafllyl are art-recognized and refer to trifluoromethanesulfonyl, *p*-toluenesulfonyl, methanesulfonyl, and nonafluorobutanesulfonyl groups, respectively. The terms triflate, tosylate, mesylate, and nonaflate are art-recognized and refer to trifluoromethanesulfonate ester, *p*-toluenesulfonate ester, methanesulfonate ester, and nonafluorobutanesulfonate ester functional groups and molecules that contain said groups, respectively.

The abbreviations Me, Et, Ph, Tf, Nf, Ts, Ms represent methyl, ethyl, phenyl, trifluoromethanesulfonyl, nonafluorobutanesulfonyl, *p*-toluenesulfonyl and methanesulfonyl, respectively. A more comprehensive list of the abbreviations utilized by organic chemists of ordinary skill in the art appears in the first issue of each volume of the *Journal of Organic Chemistry*; this list is typically presented in a table entitled Standard List of Abbreviations. The abbreviations contained in said list, and all abbreviations utilized by organic chemists of ordinary skill in the art are hereby incorporated by reference.

Certain compounds of the present invention may exist in particular geometric or stereoisomeric forms. The present invention contemplates all such compounds, including cis- and trans-isomers, *R*- and *S*-enantiomers, diastereomers, (D)-isomers, (L)-isomers, the racemic mixtures thereof, and other mixtures thereof, as falling within the scope of the invention. Additional asymmetric carbon atoms may be present in a substituent such as an alkyl group. All such isomers, as well as mixtures thereof, are intended to be included in this invention.

If, for instance, a particular enantiomer of a compound of the present invention is desired, it may be prepared by asymmetric synthesis, or by derivation with a chiral auxiliary, where the resulting diastereomeric mixture is separated and the auxiliary group cleaved to provide the pure desired enantiomers. Alternatively, where the molecule

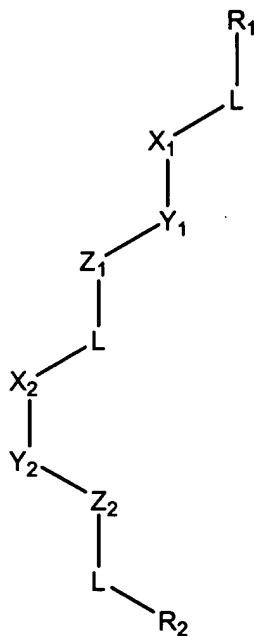
contains a basic functional group, such as amino, or an acidic functional group, such as carboxyl, diastereomeric salts may be formed with an appropriate optically active acid or base, followed by resolution of the diastereomers thus formed by fractional crystallization or chromatographic means well known in the art, and subsequent recovery of the pure enantiomers.

Contemplated equivalents of the compounds described above include compounds which otherwise correspond thereto, and which have the same general properties thereof (e.g., the ability to inhibit hedgehog signaling), wherein one or more simple variations of substituents are made which do not adversely affect the efficacy of the compound. In general, the compounds of the present invention may be prepared by the methods illustrated in the general reaction schemes as, for example, described below, or by modifications thereof, using readily available starting materials, reagents and conventional synthesis procedures. In these reactions, it is also possible to make use of variants which are in themselves known, but are not mentioned here.

For purposes of this invention, the chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, Handbook of Chemistry and Physics, 67th Ed., 1986-87, inside cover. Also for purposes of this invention, the term "hydrocarbon" is contemplated to include all permissible compounds having at least one hydrogen and one carbon atom. In a broad aspect, the permissible hydrocarbons include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and nonaromatic organic compounds which can be substituted or unsubstituted.

III. Exemplary Compounds of the Invention.

As described in further detail below, it is contemplated that the subject methods can be carried out using a variety of different small molecules which can be readily identified, for example, by such drug screening assays as described herein. The variable groups and numbers (e.g., R₁, L, Z₂) are individually and distinctly defined for Formulae I and II together, and for Formulae III and IV together, and are not necessarily consistent between these two pairings. For example, compounds useful in the subject methods include compounds may be represented by general formula (I):



Formula I

wherein, as valence and stability permit,

R_1 and R_2 , independently for each occurrence, represent H, lower alkyl, aryl (e.g., substituted or unsubstituted), aralkyl (e.g., substituted or unsubstituted, e.g., $-(CH_2)_n$ aryl), or heteroaryl (e.g., substituted or unsubstituted), or heteroaralkyl (e.g., substituted or unsubstituted, e.g., $-(CH_2)_n$ heteroaralkyl-);

L , independently for each occurrence, is absent or represents $-(CH_2)_n$ -alkyl, -alkenyl-, -alkynyl-, $-(CH_2)_n$ alkenyl-, $-(CH_2)_n$ alkynyl-, $-(CH_2)_nO(CH_2)_p$ -, $-(CH_2)_nNR_2(CH_2)_p$ -, $-(CH_2)_nS(CH_2)_p$ -, $-(CH_2)_n$ alkenyl $(CH_2)_p$ -, $-(CH_2)_n$ alkynyl $(CH_2)_p$ -, $-O(CH_2)_n$ -, $-NR_2(CH_2)_n$ -, or $-S(CH_2)_n$;

X_1 and X_2 can be selected, independently, from $-N(R_8)$ -, $-O$ -, $-S$ -, $-Se$ -, $-N=N$ -, $-ON=CH$ -, $-(R_8)N-N(R_8)$ -, $-ON(R_8)$ -, a heterocycle, or a direct bond between L and Y_1 or Y_2 , respectively;

Y_1 and Y_2 can be selected, independently, from $-C(=O)$ -, $-C(=S)$ -, $-S(O_2)$ -, $-S(O)$ -, $-C(=NCN)$ -, $-P(=O)(OR_2)$ -, a heteroaromatic group, or a direct bond between X_1 and Z_1 or X_2 and Z_2 , respectively;

Z_1 and Z_2 can be selected, independently, from $-N(R_8)-$, $-O-$, $-S-$, $-Se-$, $-N=N-$, $-ON=CH-$, $-R_8N-NR_8-$, $-ONR_8-$, a heterocycle, or a direct bond between Y_1 or Y_2 , respectively, and L;

R_8 , independently for each occurrence, represents H, lower alkyl, $-(CH_2)_n$ aryl (e.g., substituted or unsubstituted), $-(CH_2)_n$ heteroaryl (e.g., substituted or unsubstituted), or two R_8 taken together may form a 4- to 8-membered ring, e.g., with X_1 and Z_1 or X_2 and Z_1 , which ring may include one or more carbonyls;

p represents, independently for each occurrence, an integer from 0 to 10, preferably from 0 to 3; and

n , individually for each occurrence, represents an integer from 0 to 10, preferably from 0 to 5.

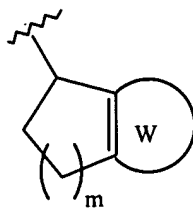
In certain embodiments, R_1 represents a substituted or unsubstituted heteroaryl group.

In certain embodiments, X_1 and X_2 can be selected from $-N(R_8)-$, $-O-$, $-S-$, a direct bond, and a heterocycle, Y_1 and Y_2 can be selected from $-C(=O)-$, $-C(=S)-$, and $-S(O_2)-$, and Z_1 or Z_2 can be selected from $-N(R_8)-$, $-O-$, $-S-$, a direct bond, and a heterocycle.

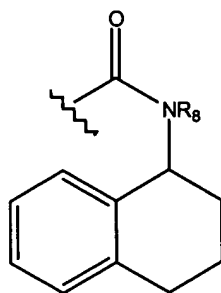
In certain related embodiments, $X_1-Y_1-Z_1$ or $X_2-Y_2-Z_2$ taken together represents a urea ($N-C(O)-N$) or an amide ($N-C(O)$ or $C(O)-N$).

In certain embodiments, X_1 or X_2 represents a diazacycarbocycle, such as a piperazine.

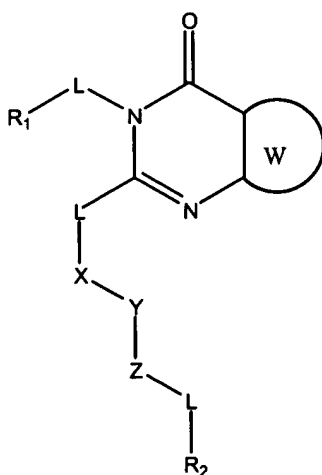
In certain embodiments, R_1 represents a fused cycloalkyl-aryl or cycloalkyl-heteroaryl system, for example:



wherein W is a substituted or unsubstituted aryl or heteroaryl ring fused to the cycloalkyl ring and m is an integer from 1-4 inclusive, e.g., from 1-3, or from 1-2. The fused system may be bound to L from any carbon of the fused system, including the position depicted above. In certain embodiments, R₁ may represent a tetrahydronaphthyl group, and preferably Y₁-X₁-L-R₁ taken together represent a tetrahydronaphthyl amide group, such as:



In embodiments wherein Y₁ and Z₁ are absent and X₁ comprises a pyrimidone, compounds useful in the present invention may be represented by general formula (II):



Formula II

wherein, as valence and stability permit,

R_1 and R_2 , independently for each occurrence, represent H, lower alkyl, $-(CH_2)_n$ aryl (e.g., substituted or unsubstituted), or $-(CH_2)_n$ heteroaryl (e.g., substituted or unsubstituted);

L, independently for each occurrence, is absent or represents $-(CH_2)_n$ -alkyl, -alkenyl-, -alkynyl-, $-(CH_2)_n$ alkenyl-, $-(CH_2)_n$ alkynyl-, $-(CH_2)_nO(CH_2)_p$ -, $-(CH_2)_nNR_2(CH_2)_p$ -, $-(CH_2)_nS(CH_2)_p$ -, $-(CH_2)_n$ alkenyl $(CH_2)_p$ -, $-(CH_2)_n$ alkynyl $(CH_2)_p$ -, $-O(CH_2)_n$ -, $-NR_2(CH_2)_n$ -, or $-S(CH_2)_n$;

X can be selected from $-N(R_8)$ -, -O-, -S-, -Se-, $-N=N$ -, $-ON=CH$ -, $-(R_8)N-N(R_8)$ -, $-ON(R_8)$ -, a heterocycle, or a direct bond between L and Y;

Y can be selected from $-C(=O)$ -, $-C(=S)$ -, $-S(O_2)$ -, $-S(O)$ -, $-C(=NCN)$ -, $-P(=O)(OR_2)$ -, a heteroaromatic group, or a direct bond between X and Z;

Z can be selected from $-N(R_8)$ -, -O-, -S-, -Se-, $-N=N$ -, $-ON=CH$ -, $-R_8N-NR_8$ -, $-ONR_8$ -, a heterocycle, or a direct bond between Y and L;

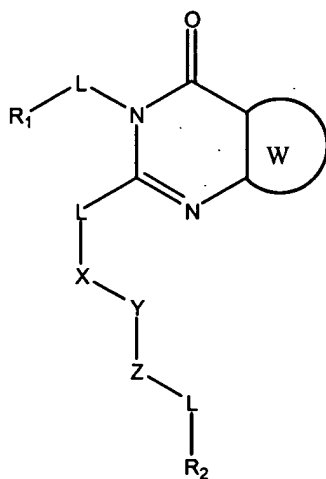
R_8 , independently for each occurrence, represents H, lower alkyl, $-(CH_2)_n$ aryl (e.g., substituted or unsubstituted), $-(CH_2)_n$ heteroaryl (e.g., substituted or unsubstituted), or two R_8 taken together may form a 4- to 8-membered ring, e.g., with X and Z, which ring may include one or more carbonyls;

W represents a substituted or unsubstituted aryl or heteroaryl ring fused to the pyrimidone ring;

p represents, independently for each occurrence, an integer from 0 to 10, preferably from 0 to 3; and

n, individually for each occurrence, represents an integer from 0 to 10, preferably from 0 to 5.

In embodiments wherein Y_1 and Z_1 are absent and X_1 comprises a pyrimidone, compounds useful in the present invention may be represented by general formula (II):



Formula II

wherein, as valence and stability permit,

R_1 and R_2 , independently for each occurrence, represent H, lower alkyl, aryl (e.g., substituted or unsubstituted), aralkyl (e.g., substituted or unsubstituted, e.g., $-(CH_2)_n$ aryl), or heteroaryl (e.g., substituted or unsubstituted), or heteroaralkyl (e.g., substituted or unsubstituted, e.g., $-(CH_2)_n$ heteroaralkyl-);

L , independently for each occurrence, is absent or represents $-(CH_2)_n$ -alkyl, -alkenyl-, -alkynyl-, $-(CH_2)_n$ alkenyl-, $-(CH_2)_n$ alkynyl-, $-(CH_2)_nO(CH_2)_p$ -, $-(CH_2)_nNR_2(CH_2)_p$ -, $-(CH_2)_nS(CH_2)_p$ -, $-(CH_2)_n$ alkenyl $(CH_2)_p$ -, $-(CH_2)_n$ alkynyl $(CH_2)_p$ -, $-O(CH_2)_n$ -, $-NR_2(CH_2)_n$ -, or $-S(CH_2)_n$ -, which may optionally be substituted with a group selected from H, substituted or unsubstituted lower alkyl, alkenyl, or alkynyl, cycloalkylalkyl (e.g., substituted or unsubstituted, e.g., $-(CH_2)_n$ cycloalkyl), (e.g., substituted or unsubstituted), aryl (e.g., substituted or unsubstituted), aralkyl (e.g., substituted or unsubstituted, e.g., $-(CH_2)_n$ aryl), or heteroaryl (e.g., substituted or unsubstituted), or heteroaralkyl (e.g., substituted or unsubstituted, e.g., $-(CH_2)_n$ heteroaralkyl-), preferably from H, lower alkyl, $-(CH_2)_n$ aryl (e.g., substituted or unsubstituted), or $-(CH_2)_n$ heteroaryl (e.g., substituted or unsubstituted);

X can be selected from $-N(R_8)-$, $-O-$, $-S-$, $-Se-$, $-N=N-$, $-ON=CH-$, $-(R_8)N-N(R_8)-$, $-ON(R_8)-$, a heterocycle, or a direct bond between L and Y;

Y can be selected from $-C(=O)-$, $-C(=S)-$, $-S(O_2)-$, $-S(O)-$, $-C(=NCN)-$, $-P(=O)(OR_2)-$, a heteroaromatic group, or a direct bond between X and Z;

Z can be selected from $-N(R_8)-$, $-O-$, $-S-$, $-Se-$, $-N=N-$, $-ON=CH-$, $-R_8N-NR_8-$, $-ONR_8-$, a heterocycle, or a direct bond between Y and L;

R_8 , independently for each occurrence, represents H, lower alkyl, aryl (e.g., substituted or unsubstituted), aralkyl (e.g., substituted or unsubstituted, e.g., $-(CH_2)_n$ aryl), or heteroaryl (e.g., substituted or unsubstituted), or heteroaralkyl (e.g., substituted or unsubstituted, e.g., $-(CH_2)_n$ heteroaralkyl-), or two R_8 taken together may form a 4- to 8-membered ring, e.g., with X and Z, which ring may include one or more carbonyls;

W represents a substituted or unsubstituted aryl or heteroaryl ring fused to the pyrimidone ring;

p represents, independently for each occurrence, an integer from 0 to 10, preferably from 0 to 3; and

n, individually for each occurrence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, R_1 represents a substituted or unsubstituted aryl or heteroaryl group, e.g., a phenyl ring, a pyridine ring, etc. In certain embodiments wherein $-LR_1$ represents a substituted aryl or heteroaryl group, R_1 is preferably not substituted with an isopropoxy (Me_2CHO-) group. In certain embodiments wherein $-LR_1$ represents a substituted aryl or heteroaryl group, R_1 is preferably not substituted with an ether group. In certain embodiments, substituents on R_1 (e.g., other than hydrogen) are selected from halogen, cyano, alkyl, alkenyl, alkynyl, aryl, hydroxyl, (unbranched alkyl-O-), silyloxy, amino, nitro, thiol, amino, imino, amido, phosphoryl, phosphonate, phosphine, carbonyl, carboxyl, carboxamide, anhydride, silyl, thioether, alkylsulfonyl, arylsulfonyl, sulfoxide,

selenoether, ketone, aldehyde, ester, or $-(CH_2)_m-R_8$. In certain embodiments, non-hydrogen substituents are selected from halogen, cyano, alkyl, alkenyl, alkynyl, aryl, nitro, thiol, imino, amido, carbonyl, carboxyl, anhydride, thioether, alkylsulfonyl, arylsulfonyl, ketone, aldehyde, and ester. In certain embodiments, non-hydrogen substituents are selected from halogen, cyano, alkyl, alkenyl, alkynyl, nitro, amido, carboxyl, anhydride, alkylsulfonyl, ketone, aldehyde, and ester.

In certain embodiments, X can be selected from $-N(R_8)-$, $-O-$, $-S-$, a direct bond, and a heterocycle, Y can be selected from $-C(=O)-$, $-C(=S)-$, and $-S(O_2)-$, and Z can be selected from $-N(R_8)-$, $-O-$, $-S-$, a direct bond, and a heterocycle. In certain such embodiments, at least one of Z and X is present.

In certain related embodiments, X-Y-Z taken together represents a urea ($NC(O)N$) or an amide ($NC(O)$ or $C(O)N$).

In certain embodiments, W is a substituted or unsubstituted benzene ring.

In certain embodiments, X represents a diazacarbycycle, such as a piperazine, e.g., substituted or unsubstituted.

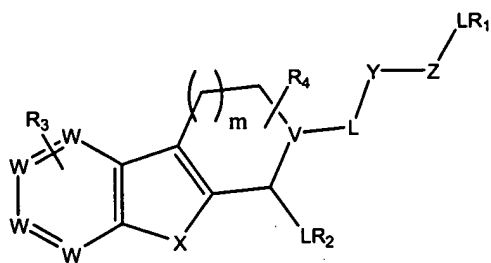
In certain embodiments, X can be selected from $-N(R_8)-$, $-O-$, $-S-$, and a direct bond, Y can be selected from $-C(=O)-$, $-C(=S)-$, and $-S(O_2)-$, and Z can be selected from $-N(R_8)-$, $-O-$, $-S-$, and a direct bond, such that at least one of X and Z is present.

In certain embodiments R_8 represents H, lower alkyl, aralkyl, heteroaralkyl, aryl, or heteroaryl, e.g., H or lower alkyl.

In certain embodiments, X represents $-NH-$.

In certain embodiments, $-L-X-$ represents $-(\text{unbranched lower alkyl})-NH-$, e.g., $-CH_2-NH-$, $-CH_2CH_2-NH-$, etc.

In certain other embodiments, compounds useful in the subject methods include compounds may be represented by general formula (III):



Formula III

wherein, as valence and stability permit,

R_1 and R_2 , independently for each occurrence, represent H, substituted or unsubstituted lower alkyl, alkenyl, or alkynyl, $-(CH_2)_n$ cycloalkyl (e.g., substituted or unsubstituted), $-(CH_2)_n$ aryl (e.g., substituted or unsubstituted), or $-(CH_2)_n$ heterocyclyl (e.g., substituted or unsubstituted);

L, independently for each occurrence, is absent or represents $-(CH_2)_n$ -alkyl, -alkenyl-, -alkynyl-, $-(CH_2)_n$ alkenyl-, $-(CH_2)_n$ alkynyl-, $-(CH_2)_nO(CH_2)_p$ -, $-(CH_2)_nNR_2(CH_2)_p$ -, $-(CH_2)_nS(CH_2)_p$ -, $-(CH_2)_n$ alkenyl $(CH_2)_p$ -, $-(CH_2)_n$ alkynyl $(CH_2)_p$ -, $-O(CH_2)_n$ -, $-NR_2(CH_2)_n$ -, or $-S(CH_2)_n$;

V represents N or CH;

W, independently for each occurrence, represents N or CH, such that preferably no more than one occurrence of W represents N;

X and Z, independently, can be selected from $-CH$ -, $-N(R_8)$ -, $-O$ -, $-S$ -, or $-Se$ -;

Y can be selected from $-C(=O)$ -, $-C(=S)$ -, $-S(O_2)$ -, $-S(O)$ -, $-C(=NCN)$ -, or $-P(=O)(OR_2)$ -;

R_8 , independently for each occurrence, represents H, substituted or unsubstituted lower alkyl, $-(CH_2)_n$ cycloalkyl (e.g., substituted or unsubstituted), $-(CH_2)_n$ aryl (e.g., substituted or unsubstituted), $-(CH_2)_n$ heterocyclyl (e.g., substituted or unsubstituted), or two R_8 taken together may form a 4- to 8-membered ring, e.g., with X_1 and Z_1 or X_2 and Z_1 , which ring may include one or more carbonyls;

R_3 and R_4 , independently represent from 1-4 substituents on the ring to which they are attached, selected from, independently for each occurrence, hydrogen, halogens, alkyls, alkenyls, alkynyls, aryls, hydroxyl, $=O$, $=S$, alkoxyl, silyloxy, amino, nitro, thiol, amines, imines, amides, phosphoryls, phosphonates, phosphines, carbonyls, carboxyls, carboxamides, anhydrides, silyls, ethers, thioethers, alkylsulfonyls, arylsulfonyls, selenoethers, ketones, aldehydes, esters, or $-(CH_2)_m-R_8$;

m represents an integer from 0-3;

p represents, independently for each occurrence, an integer from 0 to 10, preferably from 0 to 3; and

n, individually for each occurrence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, R_1 and R_2 are independently selected from substituted or unsubstituted aryl, heterocyclyl, branched or unbranched alkyl, or cycloalkyl. In embodiments wherein R_1 or R_2 is aryl or heterocyclyl, substituents are preferably selected from H, alkyl, acyl, carboxy, ester, amide, cyano, ether, thioether, amino, halogen, nitro, and trihalomethyl.

In certain embodiments, R_3 is absent or represents one or two substituents selected from alkyl, acyl, carboxy, ester, amide, cyano, ether, thioether, amino, acyl, halogen, nitro, and trihalomethyl.

In certain embodiments, R_4 is absent or represents one or two substituents selected from ether, amino, thioether, alkyl, aryl, (=O), or carbonyl (e.g., carboxy, ester, ketone, aldehyde, etc.).

In certain embodiments, L is absent for each occurrence, or represents $-CH_2-$ or $-CH_2CH_2-$.

In certain embodiments, X represents NR_8 . R_8 preferably represents H.

In certain embodiments, Z represents NR_8 . R_8 preferably represents H.

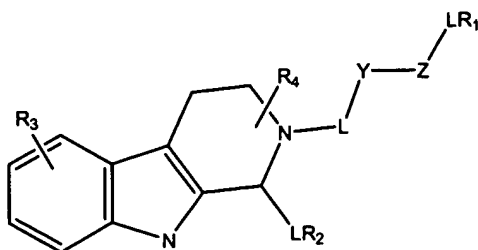
In certain embodiments, Y represents $-C(=O)-$, $-C(=S)-$, or $-S(O_2)-$.

In certain embodiments, m is 1.

In certain embodiments, W represents CH in all occurrences.

In certain embodiments, V represents N.

In certain embodiments, compounds useful in the present invention may be represented by general formula (IV):



Formula IV

wherein, as valence and stability permit,

R_1 and R_2 , independently for each occurrence, represent H, substituted or unsubstituted lower alkyl, alkenyl, or alkynyl, $-(CH_2)_n$ cycloalkyl (e.g., substituted or unsubstituted), $-(CH_2)_n$ aryl (e.g., substituted or unsubstituted), or $-(CH_2)_n$ heterocyclyl (e.g., substituted or unsubstituted);

L, independently for each occurrence, is absent or represents $-(CH_2)_n$ -alkyl, -alkenyl-, -alkynyl-, $-(CH_2)_n$ alkenyl-, $-(CH_2)_n$ alkynyl-, $-(CH_2)_nO(CH_2)_p$ -, $-(CH_2)_nNR_2(CH_2)_p$ -, $-(CH_2)_nS(CH_2)_p$ -, $-(CH_2)_n$ alkenyl $(CH_2)_p$ -, $-(CH_2)_n$ alkynyl $(CH_2)_p$ -, $-O(CH_2)_n$ -, $-NR_2(CH_2)_n$ -, or $-S(CH_2)_n$;

X and Z, independently, can be selected from $-CH$ -, $-N(R_8)$ -, $-O$ -, $-S$ -, or $-Se$ -;

Y can be selected from $-C(=O)$ -, $-C(=S)$ -, $-S(O_2)$ -, $-S(O)$ -, $-C(=NCN)$ -, or $-P(=O)(OR_2)$ -;

R_8 , independently for each occurrence, represents H, substituted or unsubstituted lower alkyl, $-(CH_2)_n$ cycloalkyl (e.g., substituted or unsubstituted), $-(CH_2)_n$ aryl (e.g., substituted or unsubstituted), $-(CH_2)_n$ heterocyclyl (e.g., substituted or unsubstituted), or two R_8 taken together may form a 4- to 8-membered ring, e.g., with X_1 and Z_1 or X_2 and Z_1 , which ring may include one or more carbonyls;

R_3 and R_4 , independently represent from 1-4 substituents on the ring to which they are attached, selected from, independently for each occurrence, hydrogen, halogens, alkyls, alkenyls, alkynyls, aryls, hydroxyl, $=O$, $=S$, alkoxy, silyloxy, amino, nitro, thiol, amines, imines, amides, phosphoryls, phosphonates, phosphines, carbonyls, carboxyls, carboxamides, anhydrides, silyls, ethers, thioethers, alkylsulfonyls, arylsulfonyls, selenoethers, ketones, aldehydes, esters, or $-(CH_2)_m-R_8$;

p represents, independently for each occurrence, an integer from 0 to 10, preferably from 0 to 3; and

n, individually for each occurrence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, R_1 and R_2 are independently selected from substituted or unsubstituted aryl, heterocyclyl, branched or unbranched alkyl, or cycloalkyl. In embodiments wherein R_1 or R_2 is aryl or heterocyclyl, substituents are preferably selected

from H, alkyl, acyl, carboxy, ester, amide, cyano, ether, thioether, amino, halogen, nitro, and trihalomethyl.

In certain embodiments, R_3 is absent or represents one or two substituents selected from alkyl, acyl, carboxy, ester, amide, cyano, ether, thioether, amino, acyl, halogen, nitro, and trihalomethyl.

In certain embodiments, R_4 is absent or represents one or two substituents selected from ether, amino, thioether, alkyl, aryl, (=O), or carbonyl (e.g., carboxy, ester, ketone, aldehyde, etc.).

In certain embodiments, L is absent for each occurrence, or represents $-CH_2-$ or $-CH_2CH_2-$.

In certain embodiments, X represents NR_8 . R_8 preferably represents H.

In certain embodiments, Z represents NR_8 . R_8 preferably represents H.

In certain embodiments, Y represents $-C(=O)-$, $-C(=S)-$, or $-S(O_2)-$.

In certain embodiments, the subject antagonists can be chosen on the basis of their selectivity for the *hedgehog* pathway. This selectivity can be for the *hedgehog* pathway versus other pathways, or for selectivity between particular *hedgehog* pathways, e.g., e.g., *ptc-1*, *ptc-2*, etc.

In certain preferred embodiments, the subject inhibitors inhibit *hedgehog*-mediated signal transduction with an ED_{50} of 1 mM or less, more preferably of 1 μ M or less, and even more preferably of 1 nM or less.

In certain preferred embodiments, the subject inhibitors inhibit *hedgehog*-mediated signal transduction with an IC_{50} of 1 mM or less, more preferably of 1 μ M or less, and even more preferably of 1 nM or less.

In particular embodiments, the small molecule is chosen for use because it is more selective for one patched isoform over the next, e.g., 10 fold, and more preferably at least 100 or even 1000 fold more selective for one patched pathway (*ptc-1*, *ptc-2*) over another.

In certain embodiments, a compound which is an antagonist of the *hedgehog* pathway is chosen to selectively antagonize *hedgehog* activity over protein kinases other than PKA, such as PKC, e.g., the compound modulates the activity of the *hedgehog* pathway at least an order of magnitude more strongly than it modulates the activity of another protein kinase, preferably at least two orders of magnitude more strongly, even more preferably at least three orders of magnitude more strongly. Thus, for example, a preferred inhibitor of the *hedgehog* pathway may inhibit *hedgehog* activity with a K_i at least an order of magnitude lower than its K_i for inhibition of PKC, preferably at least two orders of magnitude lower, even more preferably at least three orders of magnitude lower. In certain embodiments, the K_i for PKA inhibition is less than 10 nM, preferably less than 1 nM, even more preferably less than 0.1 nM.

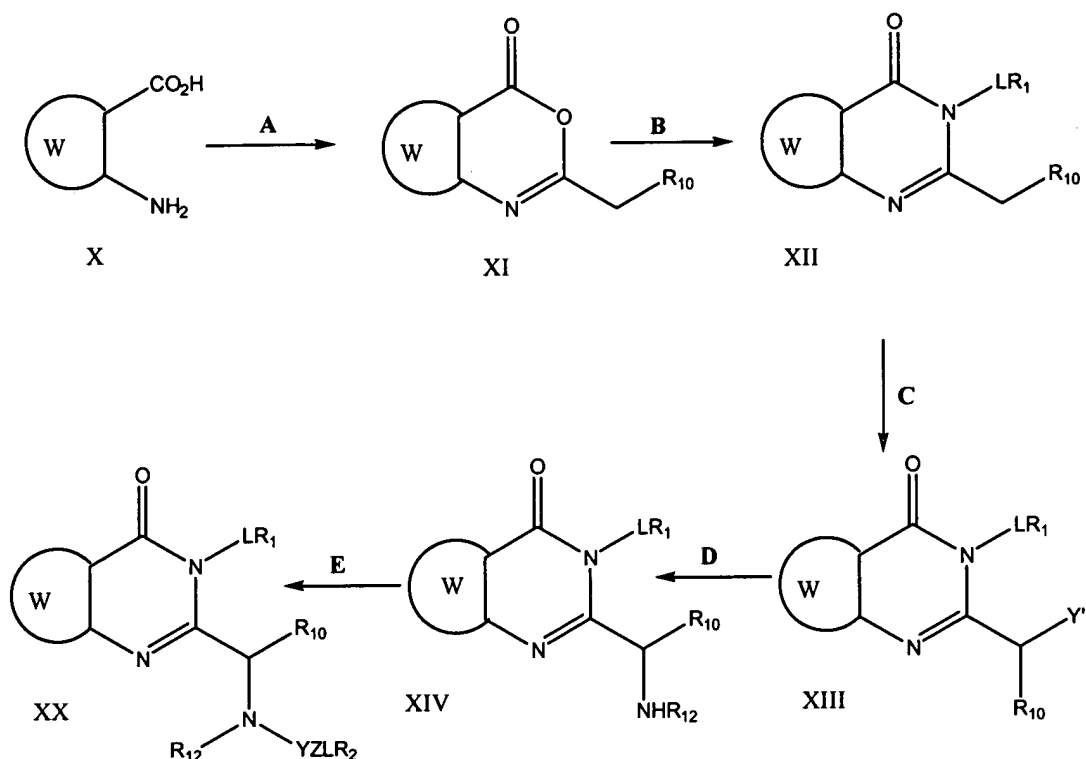
In certain embodiments, a subject compound binds to *patched*, and inhibits *hedgehog* signalling (e.g., lowers *gli* transcription by at least 5%, 10%, or more). In certain embodiments, such a compound has a structure other than those of Formulae I and II, above.

Synthesis of Subject Compounds

In certain embodiments, the present invention relates to techniques and approaches for the synthesis of subject compounds as described by Formulae I-VIII.

For example, in certain embodiments, the present invention relates to a method for preparing a subject compound as set forth in Scheme I,

Scheme I



wherein step (A) comprises reacting a compound having a structure of Formula X, wherein W represents a substituted or unsubstituted aryl or heteroaryl ring, such as a benzene ring, having an amino group and a carboxylic acid group in adjacent (*ortho*) positions, with an acylating agent having the formula $R_{10}CH_2C(=O)X'$, wherein R_{10} , independently for each occurrence, represents substituted or unsubstituted alkyl, alkenyl, cycloalkyl, cycloalkylalkyl, aralkyl, or heteroaralkyl, and X' represents a halogen or $-OC(=O)CH_2R_{10}$, under conditions that produce a compound having a structure of Formula XI;

step (B) comprises reacting a compound having a structure of Formula XI with an amine having the formula R_1LNH_2 , wherein R_1 and L are as defined above, under conditions that result in a compound having a structure of Formula XII;

step (C) comprises reacting a compound having a structure of Formula XII with a halogenating agent, such as chlorine, bromine, iodine, N-bromosuccinimide, N-chlorosuccinimide, N-iodosuccinimide, $ClBr$, IBr , ClI , or a reagent that generates a halogen radical (such as $Cl\cdot$, $Br\cdot$, or $I\cdot$) under conditions that result in a compound having a structure of Formula XIII, wherein Y' represents a halogen such as Cl , Br , or I ;

step (D) comprises reacting a compound having a structure of Formula XIII with an amine having the formula H_2NR_{12} , wherein R_{12} represents a lower alkyl group or a silyl group, such as a trialkylsilyl, triarylsilyl, dialkylarylsilyl, or diarylalkylsilyl group, under conditions that result in a compound having a structure of Formula XIV; and

step (E) comprises reacting a compound having a structure of Formula XIV with a terminating group having a structure of R_2V' to produce a compound having a structure of Formula XX, wherein R_2 is as defined above, and V' represents a functional group selected from $ZC(=W)Cl$, $ZC(=W)Br$, isocyanate, isothiocyanate, $ZC(=W)WC(=W)ZR_2$, ZSO_2Cl , ZSO_2Br , $ZSOCl$, $ZSOBr$, or an activated acylating moiety prepared *in situ*.

In certain embodiments, a subject compound may be prepared by providing a compound having a structure of Formula XI and performing steps (B) to (E). In certain embodiments, a subject compound may be prepared by providing a compound having a structure of Formula XII and performing steps (C) to (E). In certain embodiments, a subject compound may be prepared by providing a compound having a structure of Formula XIII and performing steps (D) to (E). In certain embodiments, a subject compound may be prepared by providing a compound having a structure of Formula XIV and performing step (E).

In certain embodiments, step (A) may be performed using an anhydride, such as a symmetrical anhydride, having the formula $R_{10}C(=O)OC(=O)R_{10}$. In certain embodiments, the reaction may be performed using the anhydride as a solvent, e.g., the reaction mixture is substantially free of a solvent other than the anhydride. In certain embodiments, R_{10} , for both occurrences, is a lower alkyl group.

In certain embodiments, step (B) may be performed by treating a compound of Formula XI with an amine in a non-polar solvent, such as chloroform, methylene chloride, ethylene chloride, toluene, benzene, ether, tetrahydrofuran, or another non-polar solvent, or any combination thereof, followed by treating the product of that reaction with an ionic base, such as an alkali metal hydroxide or alkoxide, such as methoxide, ethoxide, etc., in a polar solvent, such as an alcoholic solvent, e.g., methanol, ethanol, propanol, ethylene glycol, propylene glycol, glycerol, etc. In certain embodiments, the conditions may include heating the reaction mixture above room temperature, e.g., to at least 50 °C,

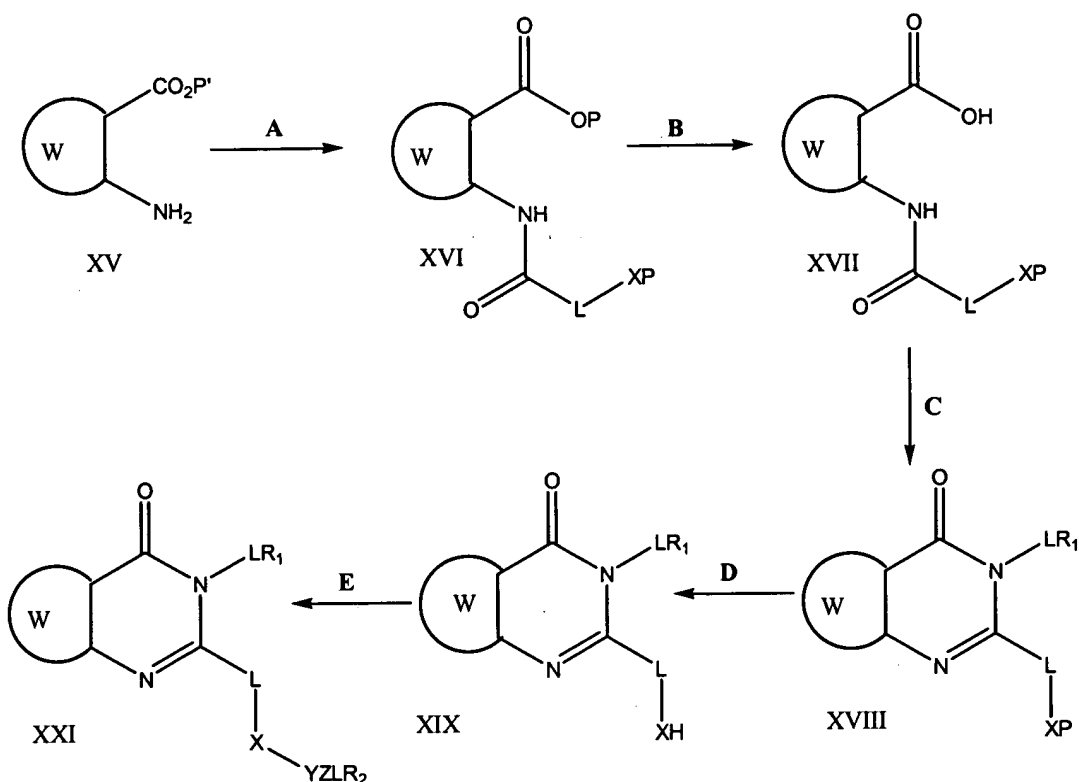
or at least 75 °C, or even at least 100 °C. In certain embodiments, this step may convert a compound of Formula X to a compound of Formula XI in at least about 80% yield, or at least about 85% yield.

In certain embodiments, step (C) may be performed using a halogen as a halogenating agent, and may be performed in a reaction mixture including a carboxylic acid, such as acetic acid or propionic acid. In certain embodiments, a mild base, such as a carboxylic acid salt, may be added to or present in the reaction mixture.

In certain embodiments, step (D) may be performed using a polar solvent, such as water, ethanol, methanol, ethylene glycol, DMF, or another polar solvent, or any combination of polar solvents. In certain embodiments, the solvent or solvent mixture comprises less than about 50% water, or is non-aqueous, i.e., is substantially free of water. In certain embodiments, the polar solvent comprises an alcohol, such as methanol, ethanol, propanol, isopropanol, butanol, isobutanol, t-butanol, sec-butanol, ethylene glycol, and 1,3-propanediol.

In certain embodiments, step (E) may be performed by generating an acylating agent *in situ*, for example, by reacting a carboxylic acid with an activating agent, such as a carbodiimide (e.g., diisopropylcarbodiimide, dicyclohexylcarbodiimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide, etc.), phosphorous-based reagents (such as BOP-Cl, PyBROP, etc.), oxalyl chloride, phosgene, triphosgene, or any other reagent that reacts with a carboxylic acid group resulting in a reactive intermediate having an increased susceptibility, relative to the carboxylic acid, towards coupling with an amine. A wide variety of such reagents are well known in the art of organic synthesis, especially peptide coupling. Similarly, a primary amine can be treated with a phosgene equivalent, such as carbonyl diimidazole, phosgene, triphosgene, diphosgene, etc., or a thiophosgene equivalent, such as thiophosgene, thiocarbonyldiimidazole, etc., to generate an acylating agent (e.g., an isocyanate, isothiocyanate, chloroformamide, or chlorothioformamide, for example) capable of reacting with an amine to form a urea or thiourea, without necessitating isolation or purification of the acylating agent.

In certain embodiments, the present invention relates to a method for preparing a subject compound as set forth in Scheme II,



wherein step (A) comprises reacting a compound having a structure of Formula XV, wherein P' represents H or a protecting group, W represents a substituted or unsubstituted aryl or heteroaryl ring, such as a benzene ring, having an amino group and a carboxylic acid or ester group in adjacent (*ortho*) positions, with an acylating agent having the formula $PXLC(=O)X'$, wherein X and L are as defined above, P represents a protecting group, and X' represents a halogen, $-OC(=O)LXP$, or a functional group generated by reacting a carboxyl group with an activating agent, such as a carbodiimide (e.g., diisopropylcarbodiimide, dicyclohexylcarbodiimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide, etc.), phosphorous-based reagents (such as BOP-Cl, PyBROP, etc.), oxalyl chloride, phosgene, triphosgene, carbonyldiimidazole, or any other reagent that reacts with a carboxylic acid group resulting in a reactive intermediate having an increased susceptibility, relative to the carboxylic acid, towards coupling with an amine, under conditions that produce a compound having a structure of Formula XVI;

step (B) comprises deprotecting the ester of a compound having a structure of Formula XVI to produce a carboxylic acid having a structure of Formula XVII, if necessary;

step (C) comprises reacting a compound having a structure of Formula XVII with an amine having the formula R_1LNH_2 , wherein R_1 and L are as defined above, under conditions that result in a compound having a structure of Formula XVIII;

step (D) comprises removing the protecting group P from a compound having a structure of Formula XVIII to generate a compound having a structure of Formula XIX;

step (E) comprises reacting a compound having a structure of Formula XIX with a terminating group having a structure of R_2Y' to produce a compound having a structure of Formula XXI, wherein R_2 is as defined above, and Y' represents a functional group selected from $ZC(=W)Cl$, $ZC(=W)Br$, isocyanate, isothiocyanate, $ZC(=W)WC(=W)ZR_2$, ZSO_2Cl , ZSO_2Br , $ZSOCl$, $ZSOBr$, or an active acylating moiety prepared *in situ*.

In certain embodiments, a subject compound may be prepared by providing a compound having a structure of Formula XVI and performing steps (B) to (E). In certain embodiments, a subject compound may be prepared by providing a compound having a structure of Formula XVII and performing steps (C) to (E). In certain embodiments, a subject compound may be prepared by providing a compound having a structure of Formula XVIII and performing steps (D) to (E). In certain embodiments, a subject compound may be prepared by providing a compound having a structure of Formula XIX and performing step (E).

In certain embodiments, step (B) may be performed by treating a compound of Formula XVI with an acid in the presence of water, or with a hydroxide base (e.g., to hydrolyze or saponify an ester), by hydrogenolysis (e.g., to remove benzyl or allyl esters), in the presence of a mild base (e.g., to remove a fluorenylmethyl ester), by adding a Lewis acid (e.g., to remove a methoxymethyl ester), in the presence of fluoride ion (e.g., to remove a 2-silylethyl ester), or any other suitable means.

In certain embodiments, step (C) may be performed by activating the carboxyl group of the compound of Formula XVII with an activating agent, such as a carbodiimide (e.g., diisopropylcarbodiimide, dicyclohexylcarbodiimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide, etc.), phosphorous-based reagents (such as BOP-Cl, PyBROP, etc.), oxalyl chloride, phosgene, triphosgene, carbonyldiimidazole, or any other reagent that reacts with a carboxylic acid group resulting in a reactive intermediate having an

increased susceptibility, relative to the carboxylic acid, towards coupling with an amine. A wide variety of such reagents are well known in the art of organic synthesis, especially peptide coupling. In certain embodiments, the conditions may include heating the reaction mixture above room temperature, e.g., to at least 50 °C, or at least 75 °C, or even at least 100 °C.

In certain embodiments, step (D) may be performed by hydrogenolysis (e.g., to remove an Alloc or CBz group), by using a base or a hydride reagent (e.g., to remove a trifluoroacetyl group), by using an acid, such as trifluoroacetic acid (e.g., to remove a BOC group), or by any other means suitable to deprotect the amine.

In certain embodiments, step (E) may be performed by generating an acylating agent *in situ*, for example, by reacting a carboxylic acid with an activating agent, such as a carbodiimide (e.g., diisopropylcarbodiimide, dicyclohexylcarbodiimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide, etc.), phosphorous-based reagents (such as BOP-Cl, PyBROP, etc.), oxalyl chloride, phosgene, triphosgene, or any other reagent that reacts with a carboxylic acid group resulting in a reactive intermediate having an increased susceptibility, relative to the carboxylic acid, towards coupling with an amine. A wide variety of such reagents are well known in the art of organic synthesis, especially peptide coupling. Similarly, a primary amine can be treated with a phosgene equivalent, such as carbonyl diimidazole, phosgene, triphosgene, diphosgene, etc., or a thiophosgene equivalent, such as thiophosgene, thiocarbonyldiimidazole, etc., to generate an acylating agent (e.g., an isocyanate, isothiocyanate, chloroformamide, or chlorothioformamide, for example) capable of reacting with an amine to form a urea or thiourea, without necessitating isolation or purification of the acylating agent.

IV. Exemplary Applications of Method and Compositions

Another aspect of the present invention relates to a method of modulating a differentiated state, survival, and/or proliferation of a cell having a *hedgehog* gain-of-function phenotype by contacting the cells with a *hedgehog* antagonist according to the subject method and as the circumstances may warrant.

For instance, it is contemplated by the invention that, in light of the findings of an apparently broad involvement of *hedgehog*, *ptc*, and *smoothed* in the formation of ordered spatial arrangements of differentiated tissues in vertebrates, the subject method could be used as part of a process for generating and/or maintaining an array of different vertebrate tissue both *in vitro* and *in vivo*. The *hedgehog* antagonist, whether inductive or anti-inductive with respect proliferation or differentiation of a given tissue, can be, as appropriate, any of the preparations described above.

For example, the present method is applicable to cell culture techniques wherein, whether for genetic or biochemical reasons, the cells have a *hedgehog* gain-of-function phenotype. *In vitro* neuronal culture systems have proved to be fundamental and indispensable tools for the study of neural development, as well as the identification of neurotrophic factors such as nerve growth factor (NGF), ciliary trophic factors (CNTF), and brain derived neurotrophic factor (BDNF). One use of the present method may be in cultures of neuronal stem cells, such as in the use of such cultures for the generation of new neurons and glia. In such embodiments of the subject method, the cultured cells can be contacted with a *hedgehog* antagonist of the present invention in order to alter the rate of proliferation of neuronal stem cells in the culture and/or alter the rate of differentiation, or to maintain the integrity of a culture of certain terminally differentiated neuronal cells. In an exemplary embodiment, the subject method can be used to culture, for example, sensory neurons or, alternatively, motoneurons. Such neuronal cultures can be used as convenient assay systems as well as sources of implantable cells for therapeutic treatments.

According to the present invention, large numbers of non-tumorigenic neural progenitor cells can be perpetuated *in vitro* and their rate of proliferation and/or differentiation can be affected by contact with *hedgehog* antagonists of the present invention. Generally, a method is provided comprising the steps of isolating neural progenitor cells from an animal, perpetuating these cells *in vitro* or *in vivo*, preferably in the presence of growth factors, and regulating the differentiation of these cells into particular neural phenotypes, e.g., neurons and glia, by contacting the cells with a *hedgehog* antagonist.

Progenitor cells are thought to be under a tonic inhibitory influence which maintains the progenitors in a suppressed state until their differentiation is required. However, recent techniques have been provided which permit these cells to be proliferated, and unlike neurons which are terminally differentiated and therefore non-dividing, they can be produced in unlimited number and are highly suitable for transplantation into heterologous and autologous hosts with neurodegenerative diseases.

By "progenitor" it is meant an oligopotent or multipotent stem cell which is able to divide without limit and, under specific conditions, can produce daughter cells which terminally differentiate such as into neurons and glia. These cells can be used for transplantation into a heterologous or autologous host. By heterologous is meant a host other than the animal from which the progenitor cells were originally derived. By autologous is meant the identical host from which the cells were originally derived.

Cells can be obtained from embryonic, post-natal, juvenile or adult neural tissue from any animal. By any animal is meant any multicellular animal which contains nervous tissue. More particularly, is meant any fish, reptile, bird, amphibian or mammal and the like. The most preferable donors are mammals, especially mice and humans.

In the case of a heterologous donor animal, the animal may be euthanized, and the brain and specific area of interest removed using a sterile procedure. Brain areas of particular interest include any area from which progenitor cells can be obtained which will serve to restore function to a degenerated area of the host's brain. These regions include areas of the central nervous system (CNS) including the cerebral cortex, cerebellum, midbrain, brainstem, spinal cord and ventricular tissue, and areas of the peripheral nervous system (PNS) including the carotid body and the adrenal medulla. More particularly, these areas include regions in the basal ganglia, preferably the striatum which consists of the caudate and putamen, or various cell groups such as the globus pallidus, the subthalamic nucleus, the nucleus basalis which is found to be degenerated in Alzheimer's Disease patients, or the substantia nigra pars compacta which is found to be degenerated in Parkinson's Disease patients.

Human heterologous neural progenitor cells may be derived from fetal tissue obtained from elective abortion, or from a post-natal, juvenile or adult organ donor. Autologous neural tissue can be obtained by biopsy, or from patients undergoing neurosurgery in which neural tissue is removed, in particular during epilepsy surgery, and more particularly during temporal lobectomies and hippocampalectomies.

Cells can be obtained from donor tissue by dissociation of individual cells from the connecting extracellular matrix of the tissue. Dissociation can be obtained using any known procedure, including treatment with enzymes such as trypsin, collagenase and the like, or by using physical methods of dissociation such as with a blunt instrument or by mincing with a scalpel to allow outgrowth of specific cell types from a tissue. Dissociation of fetal cells can be carried out in tissue culture medium, while a preferable medium for dissociation of juvenile and adult cells is artificial cerebral spinal fluid (aCSF). Regular aCSF contains 124 mM NaCl, 5 mM KCl, 1.3 mM MgCl₂, 2 mM CaCl₂, 26 mM NaHCO₃, and 10 mM D-glucose. Low Ca²⁺ aCSF contains the same ingredients except for MgCl₂ at a concentration of 3.2 mM and CaCl₂ at a concentration of 0.1 mM.

Dissociated cells can be placed into any known culture medium capable of supporting cell growth, including MEM, DMEM, RPMI, F-12, and the like, containing supplements which are required for cellular metabolism such as glutamine and other amino acids, vitamins, minerals and useful proteins such as transferrin and the like. Medium may also contain antibiotics to prevent contamination with yeast, bacteria and fungi such as penicillin, streptomycin, gentamicin and the like. In some cases, the medium may contain serum derived from bovine, equine, chicken and the like. A particularly preferable medium for cells is a mixture of DMEM and F-12.

Conditions for culturing should be close to physiological conditions. The pH of the culture media should be close to physiological pH, preferably between pH 6-8, more preferably close to pH 7, even more particularly about pH 7.4. Cells should be cultured at a temperature close to physiological temperature, preferably between 30 °C-40 °C, more preferably between 32 °C-38 °C, and most preferably between 35 °C-37 °C.

Cells can be grown in suspension or on a fixed substrate, but proliferation of the progenitors is preferably done in suspension to generate large numbers of cells by formation of "neurospheres" (see, for example, Reynolds et al. (1992) *Science* 255:1070-1709; and PCT Publications WO93/01275, WO94/09119, WO94/10292, and WO94/16718). In the case of propagating (or splitting) suspension cells, flasks are shaken well and the neurospheres allowed to settle on the bottom corner of the flask. The spheres are then transferred to a 50 ml centrifuge tube and centrifuged at low speed. The medium is aspirated, the cells resuspended in a small amount of medium with growth factor, and the cells mechanically dissociated and resuspended in separate aliquots of media.

Cell suspensions in culture medium are supplemented with any growth factor which allows for the proliferation of progenitor cells and seeded in any receptacle capable of sustaining cells, though as set out above, preferably in culture flasks or roller bottles. Cells typically proliferate within 3-4 days in a 37 °C incubator, and proliferation can be reinitiated at any time after that by dissociation of the cells and resuspension in fresh medium containing growth factors.

In the absence of substrate, cells lift off the floor of the flask and continue to proliferate in suspension forming a hollow sphere of undifferentiated cells. After approximately 3-10 days *in vitro*, the proliferating clusters (neurospheres) are fed every 2-7 days, and more particularly every 2-4 days by gentle centrifugation and resuspension in medium containing growth factor.

After 6-7 days *in vitro*, individual cells in the neurospheres can be separated by physical dissociation of the neurospheres with a blunt instrument, more particularly by triturating the neurospheres with a pipette. Single cells from the dissociated neurospheres are suspended in culture medium containing growth factors, and differentiation of the cells can be control in culture by plating (or resuspending) the cells in the presence of a *hedgehog* antagonist.

To further illustrate other uses of the subject *hedgehog* antagonists, it is noted that intracerebral grafting has emerged as an additional approach to central nervous system therapies. For example, one approach to repairing damaged brain tissues involves the

transplantation of cells from fetal or neonatal animals into the adult brain (Dunnett et al. (1987) *J Exp Biol* 123:265-289; and Freund et al. (1985) *J Neurosci* 5:603-616). Fetal neurons from a variety of brain regions can be successfully incorporated into the adult brain, and such grafts can alleviate behavioral defects. For example, movement disorder induced by lesions of dopaminergic projections to the basal ganglia can be prevented by grafts of embryonic dopaminergic neurons. Complex cognitive functions that are impaired after lesions of the neocortex can also be partially restored by grafts of embryonic cortical cells. The subject method can be used to regulate the growth state in the culture, or where fetal tissue is used, especially neuronal stem cells, can be used to regulate the rate of differentiation of the stem cells.

Stem cells useful in the present invention are generally known. For example, several neural crest cells have been identified, some of which are multipotent and likely represent uncommitted neural crest cells, and others of which can generate only one type of cell, such as sensory neurons, and likely represent committed progenitor cells. The role of *hedgehog* antagonists employed in the present method to culture such stem cells can be to regulate differentiation of the uncommitted progenitor, or to regulate further restriction of the developmental fate of a committed progenitor cell towards becoming a terminally differentiated neuronal cell. For example, the present method can be used *in vitro* to regulate the differentiation of neural crest cells into glial cells, schwann cells, chromaffin cells, cholinergic sympathetic or parasympathetic neurons, as well as peptidergic and serotonergic neurons. The *hedgehog* antagonists can be used alone, or can be used in combination with other neurotrophic factors which act to more particularly enhance a particular differentiation fate of the neuronal progenitor cell.

In addition to the implantation of cells cultured in the presence of the subject *hedgehog* antagonists, yet another aspect of the present invention concerns the therapeutic application of a *hedgehog* antagonist to regulate the growth state of neurons and other neuronal cells in both the central nervous system and the peripheral nervous system. The ability of *ptc*, *hedgehog*, and *smoothed* to regulate neuronal differentiation during development of the nervous system and also presumably in the adult state indicates that, in certain instances, the subject *hedgehog* antagonists can be expected to facilitate control

of adult neurons with regard to maintenance, functional performance, and aging of normal cells; repair and regeneration processes in chemically or mechanically lesioned cells; and treatment of degeneration in certain pathological conditions. In light of this understanding, the present invention specifically contemplates applications of the subject method to the treatment protocol of (prevention and/or reduction of the severity of) neurological conditions deriving from: (i) acute, subacute, or chronic injury to the nervous system, including traumatic injury, chemical injury, vascular injury and deficits (such as the ischemia resulting from stroke), together with infectious/inflammatory and tumor-induced injury; (ii) aging of the nervous system including Alzheimer's disease; (iii) chronic neurodegenerative diseases of the nervous system, including Parkinson's disease, Huntington's chorea, amyotrophic lateral sclerosis and the like, as well as spinocerebellar degenerations; and (iv) chronic immunological diseases of the nervous system or affecting the nervous system, including multiple sclerosis.

As appropriate, the subject method can also be used in generating nerve prostheses for the repair of central and peripheral nerve damage. In particular, where a crushed or severed axon is intubulated by use of a prosthetic device, *hedgehog* antagonists can be added to the prosthetic device to regulate the rate of growth and regeneration of the dendritic processes. Exemplary nerve guidance channels are described in U.S. patents 5,092,871 and 4,955,892.

In another embodiment, the subject method can be used in the treatment of neoplastic or hyperplastic transformations such as may occur in the central nervous system. For instance, the *hedgehog* antagonists can be utilized to cause such transformed cells to become either post-mitotic or apoptotic. The present method may, therefore, be used as part of a treatment for, e.g., malignant gliomas, meningiomas, medulloblastomas, neuroectodermal tumors, and ependymomas.

In a preferred embodiment, the subject method can be used as part of a treatment regimen for malignant medulloblastoma and other primary CNS malignant neuroectodermal tumors.

In certain embodiments, the subject method is used as part of treatment program for medulloblastoma. Medulloblastoma, a primary brain tumor, is the most common brain tumor in children. A medulloblastoma is a primitive neuroectodermal tumor arising in the posterior fossa. They account for approximately 25% of all pediatric brain tumors (Miller). Histologically, they are small round cell tumors commonly arranged in true rosettes, but may display some differentiation to astrocytes, ependymal cells or neurons (Rorke; Kleihues). PNET's may arise in other areas of the brain including the pineal gland (pineoblastoma) and cerebrum. Those arising in the supratentorial region generally fare worse than their PF counterparts.

Medulloblastoma/PNET's are known to recur anywhere in the CNS after resection, and can even metastasize to bone. Pretreatment evaluation should therefore include an examination of the spinal cord to exclude the possibility of "dropped metastases". Gadolinium-enhanced MRI has largely replaced myelography for this purpose, and CSF cytology is obtained postoperatively as a routine procedure.

In other embodiments, the subject method is used as part of treatment program for ependymomas. Ependymomas account for approximately 10% of the pediatric brain tumors in children. Grossly, they are tumors that arise from the ependymal lining of the ventricles and microscopically form rosettes, canals, and perivascular rosettes. In the CHOP series of 51 children reported with ependymomas, $\frac{3}{4}$ were histologically benign. Approximately $\frac{2}{3}$ arose from the region of the 4th ventricle. One third presented in the supratentorial region. Age at presentation peaks between birth and 4 years, as demonstrated by SEER data as well as data from CHOP. The median age is about 5 years. Because so many children with this disease are babies, they often require multimodal therapy.

Yet another aspect of the present invention concerns the observation in the art that *ptc*, *hedgehog*, and/or *smoothed* are involved in morphogenic signals involved in other vertebrate organogenic pathways in addition to neuronal differentiation as described above, having apparent roles in other endodermal patterning, as well as both mesodermal and endodermal differentiation processes. Thus, it is contemplated by the invention that

compositions comprising *hedgehog* antagonists can also be utilized for both cell culture and therapeutic methods involving generation and maintenance of non-neuronal tissue.

In one embodiment, the present invention makes use of the discovery that *ptc*, *hedgehog*, and *smoothed* are apparently involved in controlling the development of stem cells responsible for formation of the digestive tract, liver, lungs, and other organs which derive from the primitive gut. *Shh* serves as an inductive signal from the endoderm to the mesoderm, which is critical to gut morphogenesis. Therefore, for example, *hedgehog* antagonists of the instant method can be employed for regulating the development and maintenance of an artificial liver which can have multiple metabolic functions of a normal liver. In an exemplary embodiment, the subject method can be used to regulate the proliferation and differentiation of digestive tube stem cells to form hepatocyte cultures which can be used to populate extracellular matrices, or which can be encapsulated in biocompatible polymers, to form both implantable and extracorporeal artificial livers.

In another embodiment, therapeutic compositions of *hedgehog* antagonists can be utilized in conjunction with transplantation of such artificial livers, as well as embryonic liver structures, to regulate uptake of intraperitoneal implantation, vascularization, and *in vivo* differentiation and maintenance of the engrafted liver tissue.

In yet another embodiment, the subject method can be employed therapeutically to regulate such organs after physical, chemical or pathological insult. For instance, therapeutic compositions comprising *hedgehog* antagonists can be utilized in liver repair subsequent to a partial hepatectomy.

The generation of the pancreas and small intestine from the embryonic gut depends on intercellular signalling between the endodermal and mesodermal cells of the gut. In particular, the differentiation of intestinal mesoderm into smooth muscle has been suggested to depend on signals from adjacent endodermal cells. One candidate mediator of endodermally derived signals in the embryonic hindgut is Sonic hedgehog. See, for example, Apelqvist et al. (1997) Curr Biol 7:801-4. The *Shh* gene is expressed throughout the embryonic gut endoderm with the exception of the pancreatic bud

endoderm, which instead expresses high levels of the homeodomain protein *Ipfl/Pdx1* (insulin promoter factor 1/pancreatic and duodenal homeobox 1), an essential regulator of early pancreatic development. Apelqvist et al., supra, have examined whether the differential expression of *Shh* in the embryonic gut tube controls the differentiation of the surrounding mesoderm into specialised mesoderm derivatives of the small intestine and pancreas. To test this, they used the promoter of the *Ipfl/Pdx1* gene to selectively express *Shh* in the developing pancreatic epithelium. In *Ipfl/Pdx1*- *Shh* transgenic mice, the pancreatic mesoderm developed into smooth muscle and interstitial cells of Cajal, characteristic of the intestine, rather than into pancreatic mesenchyme and spleen. Also, pancreatic explants exposed to *Shh* underwent a similar program of intestinal differentiation. These results provide evidence that the differential expression of endodermally derived *Shh* controls the fate of adjacent mesoderm at different regions of the gut tube.

In the context of the present invention, it is contemplated therefore that the subject *hedgehog* antagonists can be used to control or regulate the proliferation and/or differentiation of pancreatic tissue both *in vivo* and *in vitro*.

There are a wide variety of pathological cell proliferative and differentiative conditions for which the inhibitors of the present invention may provide therapeutic benefits, with the general strategy being, for example, the correction of aberrant insulin expression, or modulation of differentiation. More generally, however, the present invention relates to a method of inducing and/or maintaining a differentiated state, enhancing survival and/or affecting proliferation of pancreatic cells, by contacting the cells with the subject inhibitors. For instance, it is contemplated by the invention that, in light of the apparent involvement of *ptc*, *hedgehog*, and *smoothened* in the formation of ordered spatial arrangements of pancreatic tissues, the subject method could be used as part of a technique to generate and/or maintain such tissue both *in vitro* and *in vivo*. For instance, modulation of the function of *hedgehog* can be employed in both cell culture and therapeutic methods involving generation and maintenance β -cells and possibly also for non-pancreatic tissue, such as in controlling the development and maintenance of

tissue from the digestive tract, spleen, lungs, urogenital organs (e.g., bladder), and other organs which derive from the primitive gut.

In an exemplary embodiment, the present method can be used in the treatment of hyperplastic and neoplastic disorders effecting pancreatic tissue, particularly those characterized by aberrant proliferation of pancreatic cells. For instance, pancreatic cancers are marked by abnormal proliferation of pancreatic cells which can result in alterations of insulin secretory capacity of the pancreas. For instance, certain pancreatic hyperplasias, such as pancreatic carcinomas, can result in hypoinsulinemia due to dysfunction of β -cells or decreased islet cell mass. To the extent that aberrant *ptc*, *hedgehog*, and *smoothened* signaling may be indicated in disease progression, the subject inhibitors, can be used to enhance regeneration of the tissue after anti-tumor therapy.

Moreover, manipulation of *hedgehog* signaling properties at different points may be useful as part of a strategy for reshaping/repairing pancreatic tissue both *in vivo* and *in vitro*. In one embodiment, the present invention makes use of the apparent involvement of *ptc*, *hedgehog*, and *smoothened* in regulating the development of pancreatic tissue. In general, the subject method can be employed therapeutically to regulate the pancreas after physical, chemical or pathological insult. In yet another embodiment, the subject method can be applied to cell culture techniques, and in particular, may be employed to enhance the initial generation of prosthetic pancreatic tissue devices. Manipulation of proliferation and differentiation of pancreatic tissue, for example, by altering *hedgehog* activity, can provide a means for more carefully controlling the characteristics of a cultured tissue. In an exemplary embodiment, the subject method can be used to augment production of prosthetic devices which require β -islet cells, such as may be used in the encapsulation devices described in, for example, the Aebischer et al. U.S. Patent No. 4,892,538, the Aebischer et al. U.S. Patent No. 5,106,627, the Lim U.S. Patent No. 4,391,909, and the Sefton U.S. Patent No. 4,353,888. Early progenitor cells to the pancreatic islets are multipotential, and apparently coactivate all the islet-specific genes from the time they first appear. As development proceeds, expression of islet-specific hormones, such as insulin, becomes restricted to the pattern of expression characteristic of mature islet cells. The phenotype of mature islet cells, however, is not stable in culture,

as reappearance of embryonal traits in mature β -cells can be observed. By utilizing the subject hedgehog antagonists, the differentiation path or proliferative index of the cells can be regulated.

Furthermore, manipulation of the differentiative state of pancreatic tissue can be utilized in conjunction with transplantation of artificial pancreas so as to promote implantation, vascularization, and *in vivo* differentiation and maintenance of the engrafted tissue. For instance, manipulation of *hedgehog* function to affect tissue differentiation can be utilized as a means of maintaining graft viability.

Bellusci et al. (1997) Development 124:53 report that *Sonic hedgehog* regulates lung mesenchymal cell proliferation *in vivo*. Accordingly, the present method can be used to regulate regeneration of lung tissue, e.g., in the treatment of emphysema.

Fujita et al. (1997) *Biochem Biophys Res Commun* 238:658 reported that Sonic hedgehog is expressed in human lung squamous carcinoma and adenocarcinoma cells. The expression of Sonic hedgehog was also detected in the human lung squamous carcinoma tissues, but not in the normal lung tissue of the same patient. They also observed that Sonic hedgehog stimulates the incorporation of BrdU into the carcinoma cells and stimulates their cell growth, while anti-Shh-N inhibited their cell growth. These results suggest that a *ptc*, *hedgehog*, and/or *smoothed* is involved in the cell growth of such transformed lung tissue and therefore indicates that the subject method can be used as part of a treatment of lung carcinoma and adenocarcinomas, and other proliferative disorders involving the lung epithelia.

Many other tumors may, based on evidence such as involvement of the hedgehog pathway in these tumors, or detected expression of hedgehog or its receptor in these tissues during development, be affected by treatment with the subject compounds. Such tumors include, but are by no means limited to, tumors related to Gorlin's syndrome (e.g., medulloblastoma, meningioma, etc.), tumors evidenced in *pct* knock-out mice (e.g., hemangioma, rhabdomyosarcoma, etc.), tumors resulting from *gli-1* amplification (e.g., glioblastoma, sarcoma, etc.), tumors connected with TRC8, a *ptc* homolog (e.g., renal carcinoma, thyroid carcinoma, etc.), *Ext-1*-related tumors (e.g., bone cancer, etc.), Shh-induced tumors (e.g., lung cancer, chondrosarcomas, etc.), and other tumors (e.g., breast

cancer, urogenital cancer (e.g., kidney, bladder, ureter, prostate, etc.), adrenal cancer, gastrointestinal cancer (e.g., stomach, intestine, etc.), etc.).

In still another embodiment of the present invention, compositions comprising *hedgehog* antagonists can be used in the *in vitro* generation of skeletal tissue, such as from skeletogenic stem cells, as well as the *in vivo* treatment of skeletal tissue deficiencies. The present invention particularly contemplates the use of *hedgehog* antagonists to regulate the rate of chondrogenesis and/or osteogenesis. By "skeletal tissue deficiency", it is meant a deficiency in bone or other skeletal connective tissue at any site where it is desired to restore the bone or connective tissue, no matter how the deficiency originated, e.g. whether as a result of surgical intervention, removal of tumor, ulceration, implant, fracture, or other traumatic or degenerative conditions.

For instance, the method of the present invention can be used as part of a regimen for restoring cartilage function to a connective tissue. Such methods are useful in, for example, the repair of defects or lesions in cartilage tissue which is the result of degenerative wear such as that which results in arthritis, as well as other mechanical derangements which may be caused by trauma to the tissue, such as a displacement of torn meniscus tissue, meniscectomy, a laxation of a joint by a torn ligament, malignment of joints, bone fracture, or by hereditary disease. The present reparative method is also useful for remodeling cartilage matrix, such as in plastic or reconstructive surgery, as well as periodontal surgery. The present method may also be applied to improving a previous reparative procedure, for example, following surgical repair of a meniscus, ligament, or cartilage. Furthermore, it may prevent the onset or exacerbation of degenerative disease if applied early enough after trauma.

In one embodiment of the present invention, the subject method comprises treating the afflicted connective tissue with a therapeutically sufficient amount of a *hedgehog* antagonist, particularly an antagonist selective for Indian *hedgehog* signal transduction, to regulate a cartilage repair response in the connective tissue by managing the rate of differentiation and/or proliferation of chondrocytes embedded in the tissue. Such connective tissues as articular cartilage, interarticular cartilage (menisci), costal cartilage (connecting the true ribs and the sternum), ligaments, and tendons are

particularly amenable to treatment in reconstructive and/or regenerative therapies using the subject method. As used herein, regenerative therapies include treatment of degenerative states which have progressed to the point of which impairment of the tissue is obviously manifest, as well as preventive treatments of tissue where degeneration is in its earliest stages or imminent.

In an illustrative embodiment, the subject method can be used as part of a therapeutic intervention in the treatment of cartilage of a diarthroidal joint, such as a knee, an ankle, an elbow, a hip, a wrist, a knuckle of either a finger or toe, or a tempomandibular joint. The treatment can be directed to the meniscus of the joint, to the articular cartilage of the joint, or both. To further illustrate, the subject method can be used to treat a degenerative disorder of a knee, such as which might be the result of traumatic injury (e.g., a sports injury or excessive wear) or osteoarthritis. The subject antagonists may be administered as an injection into the joint with, for instance, an arthroscopic needle. In some instances, the injected agent can be in the form of a hydrogel or other slow release vehicle described above in order to permit a more extended and regular contact of the agent with the treated tissue.

The present invention further contemplates the use of the subject method in the field of cartilage transplantation and prosthetic device therapies. However, problems arise, for instance, because the characteristics of cartilage and fibrocartilage varies between different tissue: such as between articular, meniscal cartilage, ligaments, and tendons, between the two ends of the same ligament or tendon, and between the superficial and deep parts of the tissue. The zonal arrangement of these tissues may reflect a gradual change in mechanical properties, and failure occurs when implanted tissue, which has not differentiated under those conditions, lacks the ability to appropriately respond. For instance, when meniscal cartilage is used to repair anterior cruciate ligaments, the tissue undergoes a metaplasia to pure fibrous tissue. By regulating the rate of chondrogenesis, the subject method can be used to particularly address this problem, by helping to adaptively control the implanted cells in the new environment and effectively resemble hypertrophic chondrocytes of an earlier developmental stage of the tissue.

In similar fashion, the subject method can be applied to enhancing both the generation of prosthetic cartilage devices and to their implantation. The need for improved treatment has motivated research aimed at creating new cartilage that is based on collagen-glycosaminoglycan templates (Stone et al. (1990) *Clin Orthop Relat Res* 252:129), isolated chondrocytes (Grande et al. (1989) *J Orthop Res* 7:208; and Takigawa et al. (1987) *Bone Miner* 2:449), and chondrocytes attached to natural or synthetic polymers (Walitani et al. (1989) *J Bone Jt Surg* 71B:74; Vacanti et al. (1991) *Plast Reconstr Surg* 88:753; von Schroeder et al. (1991) *J Biomed Mater Res* 25:329; Freed et al. (1993) *J Biomed Mater Res* 27:11; and the Vacanti et al. U.S. Patent No. 5,041,138). For example, chondrocytes can be grown in culture on biodegradable, biocompatible highly porous scaffolds formed from polymers such as polyglycolic acid, polylactic acid, agarose gel, or other polymers which degrade over time as function of hydrolysis of the polymer backbone into innocuous monomers. The matrices are designed to allow adequate nutrient and gas exchange to the cells until engraftment occurs. The cells can be cultured *in vitro* until adequate cell volume and density has developed for the cells to be implanted. One advantage of the matrices is that they can be cast or molded into a desired shape on an individual basis, so that the final product closely resembles the patient's own ear or nose (by way of example), or flexible matrices can be used which allow for manipulation at the time of implantation, as in a joint.

In one embodiment of the subject method, the implants are contacted with a *hedgehog* antagonist during certain stages of the culturing process in order to manage the rate of differentiation of chondrocytes and the formation of hypertrophic chondrocytes in the culture.

In another embodiment, the implanted device is treated with a *hedgehog* antagonist in order to actively remodel the implanted matrix and to make it more suitable for its intended function. As set out above with respect to tissue transplants, the artificial transplants suffer from the same deficiency of not being derived in a setting which is comparable to the actual mechanical environment in which the matrix is implanted. The ability to regulate the chondrocytes in the matrix by the subject method can allow the implant to acquire characteristics similar to the tissue for which it is intended to replace.

In yet another embodiment, the subject method is used to enhance attachment of prosthetic devices. To illustrate, the subject method can be used in the implantation of a periodontal prosthesis, wherein the treatment of the surrounding connective tissue stimulates formation of periodontal ligament about the prosthesis.

In still further embodiments, the subject method can be employed as part of a regimen for the generation of bone (osteogenesis) at a site in the animal where such skeletal tissue is deficient. Indian hedgehog is particularly associated with the hypertrophic chondrocytes that are ultimately replaced by osteoblasts. For instance, administration of a *hedgehog* antagonists of the present invention can be employed as part of a method for regulating the rate of bone loss in a subject. For example, preparations comprising *hedgehog* antagonists can be employed, for example, to control endochondral ossification in the formation of a "model" for ossification.

In yet another embodiment of the present invention, a *hedgehog* antagonist can be used to regulate spermatogenesis. The *hedgehog* proteins, particularly Dhh, have been shown to be involved in the differentiation and/or proliferation and maintenance of testicular germ cells. Dhh expression is initiated in Sertoli cell precursors shortly after the activation of Sry (testicular determining gene) and persists in the testis into the adult. Males are viable but infertile, owing to a complete absence of mature sperm. Examination of the developing testis in different genetic backgrounds suggests that Dhh regulates both early and late stages of spermatogenesis. Bitgood et al. (1996) Curr Biol 6:298. In a preferred embodiment, the *hedgehog* antagonist can be used as a contraceptive. In similar fashion, *hedgehog* antagonists of the subject method are potentially useful for modulating normal ovarian function.

The subject method also has wide applicability to the treatment or prophylaxis of disorders afflicting epithelial tissue, as well as in cosmetic uses. In general, the method can be characterized as including a step of administering to an animal an amount of a *hedgehog* antagonist effective to alter the growth state of a treated epithelial tissue. The mode of administration and dosage regimens will vary depending on the epithelial tissue(s) which is to be treated. For example, topical formulations will be preferred where the treated tissue is epidermal tissue, such as dermal or mucosal tissues.

A method which "promotes the healing of a wound" results in the wound healing more quickly as a result of the treatment than a similar wound heals in the absence of the treatment. "Promotion of wound healing" can also mean that the method regulates the proliferation and/or growth of, *inter alia*, keratinocytes, or that the wound heals with less scarring, less wound contraction, less collagen deposition and more superficial surface area. In certain instances, "promotion of wound healing" can also mean that certain methods of wound healing have improved success rates, (e.g., the take rates of skin grafts,) when used together with the method of the present invention.

Despite significant progress in reconstructive surgical techniques, scarring can be an important obstacle in regaining normal function and appearance of healed skin. This is particularly true when pathologic scarring such as keloids or hypertrophic scars of the hands or face causes functional disability or physical deformity. In the severest circumstances, such scarring may precipitate psychosocial distress and a life of economic deprivation. Wound repair includes the stages of hemostasis, inflammation, proliferation, and remodeling. The proliferative stage involves multiplication of fibroblasts and endothelial and epithelial cells. Through the use of the subject method, the rate of proliferation of epithelial cells in and proximal to the wound can be controlled in order to accelerate closure of the wound and/or minimize the formation of scar tissue.

The present treatment can also be effective as part of a therapeutic regimen for treating oral and paraoral ulcers, e.g. resulting from radiation and/or chemotherapy. Such ulcers commonly develop within days after chemotherapy or radiation therapy. These ulcers usually begin as small, painful irregularly shaped lesions usually covered by a delicate gray necrotic membrane and surrounded by inflammatory tissue. In many instances, lack of treatment results in proliferation of tissue around the periphery of the lesion on an inflammatory basis. For instance, the epithelium bordering the ulcer usually demonstrates proliferative activity, resulting in loss of continuity of surface epithelium. These lesions, because of their size and loss of epithelial integrity, dispose the body to potential secondary infection. Routine ingestion of food and water becomes a very painful event and, if the ulcers proliferate throughout the alimentary canal, diarrhea usually is evident with all its complicating factors. According to the present invention, a

treatment for such ulcers which includes application of an *hedgehog* antagonist can reduce the abnormal proliferation and differentiation of the affected epithelium, helping to reduce the severity of subsequent inflammatory events.

The subject method and compositions can also be used to treat wounds resulting from dermatological diseases, such as lesions resulting from autoimmune disorders such as psoriasis. Atopic dermatitis refers to skin trauma resulting from allergies associated with an immune response caused by allergens such as pollens, foods, dander, insect venoms and plant toxins.

In other embodiments, antiproliferative preparations of *hedgehog* antagonists can be used to inhibit lens epithelial cell proliferation to prevent post-operative complications of extracapsular cataract extraction. Cataract is an intractable eye disease and various studies on a treatment of cataract have been made. But at present, the treatment of cataract is attained by surgical operations. Cataract surgery has been applied for a long time and various operative methods have been examined. Extracapsular lens extraction has become the method of choice for removing cataracts. The major medical advantages of this technique over intracapsular extraction are lower incidence of aphakic cystoid macular edema and retinal detachment. Extracapsular extraction is also required for implantation of posterior chamber type intraocular lenses which are now considered to be the lenses of choice in most cases.

However, a disadvantage of extracapsular cataract extraction is the high incidence of posterior lens capsule opacification, often called after-cataract, which can occur in up to 50% of cases within three years after surgery. After-cataract is caused by proliferation of equatorial and anterior capsule lens epithelial cells which remain after extracapsular lens extraction. These cells proliferate to cause Sommerling rings, and along with fibroblasts which also deposit and occur on the posterior capsule, cause opacification of the posterior capsule, which interferes with vision. Prevention of after-cataract would be preferable to treatment. To inhibit secondary cataract formation, the subject method provides a means for inhibiting proliferation of the remaining lens epithelial cells. For example, such cells can be induced to remain quiescent by instilling a solution containing an *hedgehog* antagonist preparation into the anterior chamber of the eye after lens

removal. Furthermore, the solution can be osmotically balanced to provide minimal effective dosage when instilled into the anterior chamber of the eye, thereby inhibiting subcapsular epithelial growth with some specificity.

The subject method can also be used in the treatment of corneopathies marked by corneal epithelial cell proliferation, as for example in ocular epithelial disorders such as epithelial downgrowth or squamous cell carcinomas of the ocular surface.

Levine et al. (1997) J Neurosci 17:6277 show that hedgehog proteins can regulate mitogenesis and photoreceptor differentiation in the vertebrate retina, and Ihh is a candidate factor from the pigmented epithelium to promote retinal progenitor proliferation and photoreceptor differentiation. Likewise, Jensen et al. (1997) Development 124:363 demonstrated that treatment of cultures of perinatal mouse retinal cells with the amino-terminal fragment of Sonic hedgehog protein results in an increase in the proportion of cells that incorporate bromodeoxuridine, in total cell numbers, and in rod photoreceptors, amacrine cells and Muller glial cells, suggesting that Sonic hedgehog promotes the proliferation of retinal precursor cells. Thus, the subject method can be used in the treatment of proliferative diseases of retinal cells and regulate photoreceptor differentiation.

Yet another aspect of the present invention relates to the use of the subject method to control hair growth. Hair is basically composed of keratin, a tough and insoluble protein; its chief strength lies in its disulphide bond of cystine. Each individual hair comprises a cylindrical shaft and a root, and is contained in a follicle, a flask-like depression in the skin. The bottom of the follicle contains a finger-like projection termed the papilla, which consists of connective tissue from which hair grows, and through which blood vessels supply the cells with nourishment. The shaft is the part that extends outwards from the skin surface, whilst the root has been described as the buried part of the hair. The base of the root expands into the hair bulb, which rests upon the papilla. Cells from which the hair is produced grow in the bulb of the follicle; they are extruded in the form of fibers as the cells proliferate in the follicle. Hair "growth" refers to the formation and elongation of the hair fiber by the dividing cells.

As is well known in the art, the common hair cycle is divided into three stages: anagen, catagen and telogen. During the active phase (anagen), the epidermal stem cells of the dermal papilla divide rapidly. Daughter cells move upward and differentiate to form the concentric layers of the hair itself. The transitional stage, catagen, is marked by the cessation of mitosis of the stem cells in the follicle. The resting stage is known as telogen, where the hair is retained within the scalp for several weeks before an emerging new hair developing below it dislodges the telogen-phase shaft from its follicle. From this model it has become clear that the larger the pool of dividing stem cells that differentiate into hair cells, the more hair growth occurs. Accordingly, methods for increasing or reducing hair growth can be carried out by potentiating or inhibiting, respectively, the proliferation of these stem cells.

In certain embodiments, the subject method can be employed as a way of reducing the growth of human hair as opposed to its conventional removal by cutting, shaving, or depilation. For instance, the present method can be used in the treatment of trichosis characterized by abnormally rapid or dense growth of hair, e.g. hypertrichosis. In an exemplary embodiment, *hedgehog* antagonists can be used to manage hirsutism, a disorder marked by abnormal hairiness. The subject method can also provide a process for extending the duration of depilation.

Moreover, because a *hedgehog* antagonist will often be cytostatic to epithelial cells, rather than cytotoxic, such agents can be used to protect hair follicle cells from cytotoxic agents which require progression into S-phase of the cell-cycle for efficacy, e.g. radiation-induced death. Treatment by the subject method can provide protection by causing the hair follicle cells to become quiescent, e.g., by inhibiting the cells from entering S phase, and thereby preventing the follicle cells from undergoing mitotic catastrophe or programmed cell death. For instance, *hedgehog* antagonists can be used for patients undergoing chemo- or radiation-therapies which ordinarily result in hair loss. By inhibiting cell-cycle progression during such therapies, the subject treatment can protect hair follicle cells from death which might otherwise result from activation of cell death programs. After the therapy has concluded, the instant method can also be removed with concomitant relief of the inhibition of follicle cell proliferation.

The subject method can also be used in the treatment of folliculitis, such as folliculitis decalvans, folliculitis ulerythematosia reticulata or keloid folliculitis. For example, a cosmetic preparation of an *hedgehog* antagonist can be applied topically in the treatment of pseudofolliculitis, a chronic disorder occurring most often in the submandibular region of the neck and associated with shaving, the characteristic lesions of which are erythematous papules and pustules containing buried hairs.

In another aspect of the invention, the subject method can be used to induce differentiation and/or inhibit proliferation of epithelially derived tissue. Such forms of these molecules can provide a basis for differentiation therapy for the treatment of hyperplastic and/or neoplastic conditions involving epithelial tissue. For example, such preparations can be used for the treatment of cutaneous diseases in which there is abnormal proliferation or growth of cells of the skin.

For instance, the pharmaceutical preparations of the invention are intended for the treatment of hyperplastic epidermal conditions, such as keratosis, as well as for the treatment of neoplastic epidermal conditions such as those characterized by a high proliferation rate for various skin cancers, as for example squamous cell carcinoma. The subject method can also be used in the treatment of autoimmune diseases affecting the skin, in particular, of dermatological diseases involving morbid proliferation and/or keratinization of the epidermis, as for example, caused by psoriasis or atopic dermatosis.

Many common diseases of the skin, such as psoriasis, squamous cell carcinoma, keratoacanthoma and actinic keratosis are characterized by localized abnormal proliferation and growth. For example, in psoriasis, which is characterized by scaly, red, elevated plaques on the skin, the keratinocytes are known to proliferate much more rapidly than normal and to differentiate less completely.

In one embodiment, the preparations of the present invention are suitable for the treatment of dermatological ailments linked to keratinization disorders causing abnormal proliferation of skin cells, which disorders may be marked by either inflammatory or non-inflammatory components. To illustrate, therapeutic preparations of a *hedgehog* antagonist, e.g., which promotes quiescence or differentiation can be used to treat varying forms of psoriasis, be they cutaneous, mucosal or ungual. Psoriasis, as described above,

is typically characterized by epidermal keratinocytes which display marked proliferative activation and differentiation along a "regenerative" pathway. Treatment with an antiproliferative embodiment of the subject method can be used to reverse the pathological epidermal activation and can provide a basis for sustained remission of the disease.

A variety of other keratotic lesions are also candidates for treatment with the subject method. Actinic keratoses, for example, are superficial inflammatory premalignant tumors arising on sun-exposed and irradiated skin. The lesions are erythematous to brown with variable scaling. Current therapies include excisional and cryosurgery. These treatments are painful, however, and often produce cosmetically unacceptable scarring. Accordingly, treatment of keratosis, such as actinic keratosis, can include application, preferably topical, of a *hedgehog* antagonist composition in amounts sufficient to inhibit hyperproliferation of epidermal/epidermoid cells of the lesion.

Acne represents yet another dermatologic ailment which may be treated by the subject method. Acne vulgaris, for instance, is a multifactorial disease most commonly occurring in teenagers and young adults, and is characterized by the appearance of inflammatory and noninflammatory lesions on the face and upper trunk. The basic defect which gives rise to acne vulgaris is hypercornification of the duct of a hyperactive sebaceous gland. Hypercornification blocks the normal mobility of skin and follicle microorganisms, and in so doing, stimulates the release of lipases by *Propionobacterium acnes* and *Staphylococcus epidermidis* bacteria and *Pitrosporum ovale*, a yeast. Treatment with an antiproliferative *hedgehog* antagonist, particularly topical preparations, may be useful for preventing the transitional features of the ducts, e.g. hypercornification, which lead to lesion formation. The subject treatment may further include, for example, antibiotics, retinoids and antiandrogens.

The present invention also provides a method for treating various forms of dermatitis. Dermatitis is a descriptive term referring to poorly demarcated lesions which are either pruritic, erythematous, scaly, blistered, weeping, fissured or crusted. These lesions arise from any of a wide variety of causes. The most common types of dermatitis are atopic, contact and diaper dermatitis. For instance, seborrheic dermatitis is a chronic,

usually pruritic, dermatitis with erythema, dry, moist, or greasy scaling, and yellow crusted patches on various areas, especially the scalp, with exfoliation of an excessive amount of dry scales. The subject method can also be used in the treatment of stasis dermatitis, an often chronic, usually eczematous dermatitis. Actinic dermatitis is dermatitis that due to exposure to actinic radiation such as that from the sun, ultraviolet waves or x- or gamma-radiation. According to the present invention, the subject method can be used in the treatment and/or prevention of certain symptoms of dermatitis caused by unwanted proliferation of epithelial cells. Such therapies for these various forms of dermatitis can also include topical and systemic corticosteroids, antipuritics, and antibiotics.

For example, it is contemplated that the subject method could be used to inhibit angiogenesis. Hedgehog is known to stimulate angiogenesis. Matrigel plugs impregnated with hedgehog protein and inserted into mice evince substantial neovascularization, whereas Matrigel plugs not carrying hedgehog show comparatively little vascularization. Hedgehog protein is also capable of increasing vascularization of the normally avascular mouse cornea. The *ptc-1* gene is expressed in normal vascular tissues, including the endothelial cells of the aorta, vascular smooth muscle cells, adventitial fibroblasts of the aorta, the coronary vasculature and cardiomyocytes of the atria and ventricles. These tissues are also sensitive to hedgehog protein. Treatment with exogenous hedgehog causes upregulation of *ptc-1* expression. In addition, hedgehog proteins stimulate proliferation of vascular smooth muscle cells in vivo. Hedgehog proteins also cause fibroblasts to increase expression of angiogenic growth factors such as VEGF, bFGF, Ang-1 and Ang-2. Lastly, hedgehog proteins are known to stimulate recovery from ischemic injury and stimulate formation of collateral vessels.

Given that hedgehog promotes angiogenesis, hedgehog antagonists are expected to act as angiogenesis inhibitors, particularly in situations where some level of hedgehog signaling is necessary for angiogenesis.

Angiogenesis is fundamental to many disorders. Persistent, unregulated angiogenesis occurs in a range of disease states, tumor metastases and abnormal growths by endothelial cells. The vasculature created as a result of angiogenic processes supports

the pathological damage seen in these conditions. The diverse pathological states created due to unregulated angiogenesis have been grouped together as angiogenic dependent or angiogenic associated diseases. Therapies directed at control of the angiogenic processes could lead to the abrogation or mitigation of these diseases.

Diseases caused by, supported by or associated with angiogenesis include ocular neovascular disease, age-related macular degeneration, diabetic retinopathy, retinopathy of prematurity, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, epidemic keratoconjunctivitis, Vitamin A deficiency, contact lens overwear, atopic keratitis, superior limbic keratitis, pterygium keratitis sicca, Sjogren's, acne rosacea, phlyctenulosis, syphilis, Mycobacteria infections, lipid degeneration, chemical burns, bacterial ulcers, fungal ulcers, Herpes simplex infections, Herpes zoster infections, protozoan infections, Kaposi sarcoma, Mooren ulcer, Terrien's marginal degeneration, marginal keratolysis, rheumatoid arthritis, systemic lupus, polyarteritis, trauma, Wegeners sarcoidosis, Scleritis, Stevens Johnson disease, periphigoid radial keratotomy, corneal graph rejection, rheumatoid arthritis, osteoarthritis chronic inflammation (eg., ulcerative colitis or Crohn's disease), hemangioma, Osler-Weber-Rendu disease, and hereditary hemorrhagic telangiectasia.

In addition, angiogenesis plays a critical role in cancer. A tumor cannot expand without a blood supply to provide nutrients and remove cellular wastes. Tumors in which angiogenesis is important include solid tumors such as rhabdomyosarcomas, retinoblastoma, Ewing sarcoma, neuroblastoma, and osteosarcoma, and benign tumors such as acoustic neuroma, neurofibroma, trachoma and pyogenic granulomas. Angiogenic factors have been found associated with several solid tumors . Prevention of angiogenesis could halt the growth of these tumors and the resultant damage to the animal due to the presence of the tumor. Angiogenesis is also associated with blood-born tumors such as leukemias, any of various acute or chronic neoplastic diseases of the bone marrow in which unrestrained proliferation of white blood cells occurs, usually accompanied by anemia, impaired blood clotting, and enlargement of the lymph nodes, liver, and spleen. It is believed that angiogenesis plays a role in the abnormalities in the bone marrow that give rise to leukemia-like tumors.

In addition to tumor growth, angiogenesis is important in metastasis. Initially, angiogenesis is important in the vascularization of the tumor which allows cancerous cells to enter the blood stream and to circulate throughout the body. After the tumor cells have left the primary site, and have settled into the secondary, metastasis site, angiogenesis must occur before the new tumor can grow and expand. Therefore, prevention of angiogenesis could lead to the prevention of metastasis of tumors and possibly contain the neoplastic growth at the primary site.

Angiogenesis is also involved in normal physiological processes such as reproduction and wound healing. Angiogenesis is an important step in ovulation and also in implantation of the blastula after fertilization. Prevention of angiogenesis could be used to induce amenorrhea, to block ovulation or to prevent implantation by the blastula.

It is anticipated that the invention will be useful for the treatment and/or prevention of respiratory distress syndrome or other disorders resulting from inappropriate lung surface tension. Respiratory distress syndrome results from insufficient surfactant in the alveolae of the lungs. The lungs of vertebrates contain surfactant, a complex mixture of lipids and protein which causes surface tension to rise during lung inflation and decrease during lung deflation. During lung deflation, surfactant decreases such that there are no surface forces that would otherwise promote alveolar collapse. Aerated alveoli that have not collapsed during expiration permit continuous oxygen and carbon dioxide transport between blood and alveolar gas and require much less force to inflate during the subsequent inspiration. During inflation, lung surfactant increases surface tension as the alveolar surface area increases. A rising surface tension in expanding alveoli opposes over-inflation in those airspaces and tends to divert inspired air to less well-aerated alveoli, thereby facilitating even lung aeration.

Respiratory distress syndrome is particularly prevalent among premature infants. Lung surfactant is normally synthesized at a very low rate until the last six weeks of fetal life. Human infants born more than six weeks before the normal term of a pregnancy have a high risk of being born with inadequate amounts of lung surfactant and inadequate rates of surfactant synthesis. The more prematurely an infant is born, the more severe the surfactant deficiency is likely to be. Severe surfactant deficiency can lead to respiratory failure within a few minutes or hours of birth. The surfactant deficiency produces

progressive collapse of alveoli (atelectasis) because of the decreasing ability of the lung to expand despite maximum inspiratory effort. As a result, inadequate amounts of oxygen reach the infant's blood. RDS can occur in adults as well, typically as a consequence of failure in surfactant biosynthesis.

Lung tissue of premature infants shows high activity of the hedgehog signaling pathway. Inhibition of this pathway using hedgehog antagonists increases the formation of lamellar bodies and increases the expression of genes involved in surfactant biosynthesis. Lamellar bodies are subcellular structures associated with surfactant biosynthesis. For these reasons, treatment of premature infants with a hedgehog antagonist should stimulate surfactant biosynthesis and ameliorate RDS. In cases where adult RDS is associated with hedgehog pathway activation, treatment with hedgehog antagonists should also be effective.

It is further contemplated that the use of hedgehog antagonists may be specifically targeted to disorders where the affected tissue and/or cells evince high hedgehog pathway activation. Expression of *gli* genes is activated by the hedgehog signaling pathway, including *gli-1*, *gli-2* and *gli-3*. *gli-1* expression is most consistently correlated with hedgehog signaling activity across a wide range of tissues and disorders, while *gli-3* is somewhat less so. The *gli* genes encode transcription factors that activate expression of many genes needed to elicit the full effects of hedgehog signaling. However, the Gli-3 transcription factor can also act as a repressor of hedgehog effector genes, and therefore, expression of *gli-3* can cause a decreased effect of the hedgehog signaling pathway. Whether Gli-3 acts as a transcriptional activator or repressor depends on post-translational events, and therefore it is expected that methods for detecting the activating form (versus the repressing form) of Gli-3 protein would also be a reliable measure of hedgehog pathway activation. *gli-2* gene expression is expected to provide a reliable marker for hedgehog pathway activation. The *gli-1* gene is strongly expressed in a wide array of cancers, hyperplasias and immature lungs, and serves as a marker for the relative activation of the hedgehog pathway. In addition, tissues, such as immature lung, that have high *gli* gene expression are strongly affected by hedgehog inhibitors. Accordingly, it is contemplated that the detection of *gli* gene expression may be used as a powerful

predictive tool to identify tissues and disorders that will particularly benefit from treatment with a hedgehog antagonist.

In preferred embodiments, *gli-1* expression levels are detected, either by direct detection of the transcript or by detection of protein levels or activity. Transcripts may be detected using any of a wide range of techniques that depend primarily on hybridization of probes to the *gli-1* transcripts or to cDNAs synthesized therefrom. Well known techniques include Northern blotting, reverse-transcriptase PCR and microarray analysis of transcript levels. Methods for detecting Gli protein levels include Western blotting, immunoprecipitation, two-dimensional polyacrylamide gel electrophoresis (2D SDS-PAGE)(preferably compared against a standard wherein the position of the Gli proteins has been determined), and mass spectroscopy. Mass spectroscopy may be coupled with a series of purification steps to allow high-throughput identification of many different protein levels in a particular sample. Mass spectroscopy and 2D SDS-PAGE can also be used to identify post-transcriptional modifications to proteins including proteolytic events, ubiquitination, phosphorylation, lipid modification etc. Gli activity may also be assessed by analyzing binding to substrate DNA or in vitro transcriptional activation of target promoters. Gel shift assays, DNA footprinting assays and DNA-protein crosslinking assays are all methods that may be used to assess the presence of a protein capable of binding to Gli binding sites on DNA.

In preferred embodiments, *gli* transcript levels are measured and diseased or disordered tissues showing abnormally high *gli* levels are treated with a hedgehog antagonist. Premature lung tissue, lung cancers (e.g., adenocarcinomas, broncho-alveolar adenocarcinomas, small cell carcinomas), breast cancers (e.g., inferior ductal carcinomas, inferior lobular carcinomas, tubular carcinomas), prostate cancers (e.g., adenocarcinomas), and benign prostatic hyperplasias all show strongly elevated *gli-1* expression levels in certain cases. Accordingly, *gli-1* expression levels are a powerful diagnostic device to determine which of these tissues should be treated with a hedgehog antagonist. In addition, there is substantial correlative evidence that cancers of urothelial cells (e.g., bladder cancer, other urogenital cancers) will also have elevated *gli-1* levels in certain cases. For example, it is known that loss of heterozygosity on chromosome 9q22 is common in bladder cancers. The *ptc-1* gene is located at this position and *ptc-1* loss of

function is probably a partial cause of hyperproliferation, as in many other cancer types. Accordingly, such cancers would also show high *gli* expression and would be particularly amenable to treatment with a hedgehog antagonist.

It is anticipated that any degree of *gli* overexpression may be useful in determining that a hedgehog antagonist will be an effective therapeutic. In preferred embodiments, *gli* should be expressed at a level at least twice as high as normal. In particularly preferred embodiments, expression is four, six, eight or ten times as high as normal.

Expression of *ptc-1* and *ptc-2* is also activated by the hedgehog signaling pathway, but these genes are inferior to the *gli* genes as markers of hedgehog pathway activation. In certain tissues only one of *ptc-1* or *ptc-2* is expressed although the hedgehog pathway is highly active. For example, in testicular development, Indian hedgehog plays an important role and the hedgehog pathway is activated, but only *ptc-2* is expressed. Accordingly, these genes are individually unreliable as markers for hedgehog pathway activation, although simultaneous measurement of both genes are contemplated as a useful indicator for tissues to be treated with a hedgehog antagonist.

Ailments which may be treated by the subject method are disorders specific to non-humans, such as mange.

In still another embodiment, the subject method can be used in the treatment of human cancers, such as tumors of epithelial tissues such as the skin. For example, *hedgehog* antagonists can be employed, in the subject method, as part of a treatment for human carcinomas, adenocarcinomas, sarcomas and the like.

In another aspect, the present invention provides pharmaceutical preparations comprising *hedgehog* antagonists. The *hedgehog* antagonists for use in the subject method may be conveniently formulated for administration with a biologically acceptable medium, such as water, buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like) or suitable mixtures thereof. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists. As used herein, "biologically acceptable medium" includes any and all solvents, dispersion media, and

the like which may be appropriate for the desired route of administration of the pharmaceutical preparation. The use of such media for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the activity of the *hedgehog* antagonist, its use in the pharmaceutical preparation of the invention is contemplated. Suitable vehicles and their formulation inclusive of other proteins are described, for example, in the book *Remington's Pharmaceutical Sciences* (Remington's Pharmaceutical Sciences. Mack Publishing Company, Easton, Pa., USA 1985). These vehicles include injectable "deposit formulations".

Pharmaceutical formulations of the present invention can also include veterinary compositions, e.g., pharmaceutical preparations of the *hedgehog* antagonists suitable for veterinary uses, e.g., for the treatment of live stock or domestic animals, e.g., dogs.

Methods of introduction may also be provided by rechargeable or biodegradable devices. Various slow release polymeric devices have been developed and tested *in vivo* in recent years for the controlled delivery of drugs, including proteinacious biopharmaceuticals. A variety of biocompatible polymers (including hydrogels), including both biodegradable and non-degradable polymers, can be used to form an implant for the sustained release of a *hedgehog* antagonist at a particular target site.

The preparations of the present invention may be given orally, parenterally, topically, or rectally. They are of course given by forms suitable for each administration route. For example, they are administered in tablets or capsule form, by injection, inhalation, eye lotion, ointment, suppository, controlled release patch, etc. administration by injection, infusion or inhalation; topical by lotion or ointment; and rectal by suppositories. Oral and topical administrations are preferred.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion.

The phrases "systemic administration," "administered systemically," "peripheral administration" and "administered peripherally" as used herein mean the administration of a compound, drug or other material other than directly into the central nervous system, such that it enters the patient's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

These compounds may be administered to humans and other animals for therapy by any suitable route of administration, including orally, nasally, as by, for example, a spray, rectally, intravaginally, parenterally, intracisternally and topically, as by powders, ointments or drops, including buccally and sublingually.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically acceptable dosage forms such as described below or by other conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

The selected dosage level will depend upon a variety of factors including the activity of the particular compound of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular hedgehog antagonist employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required

in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

In general, a suitable daily dose of a compound of the invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. Generally, intravenous, intracerebroventricular and subcutaneous doses of the compounds of this invention for a patient will range from about 0.0001 to about 100 mg per kilogram of body weight per day.

If desired, the effective daily dose of the active compound may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms.

The term "treatment" is intended to encompass also prophylaxis, therapy and cure.

The patient receiving this treatment is any animal in need, including primates, in particular humans, and other mammals such as equines, cattle, swine and sheep; and poultry and pets in general.

The compound of the invention can be administered as such or in admixtures with pharmaceutically acceptable and/or sterile carriers and can also be administered in conjunction with other antimicrobial agents such as penicillins, cephalosporins, aminoglycosides and glycopeptides. Conjunctive therapy, thus includes sequential, simultaneous and separate administration of the active compound in a way that the therapeutical effects of the first administered one is not entirely disappeared when the subsequent is administered.

V. Pharmaceutical Compositions

While it is possible for a compound of the present invention to be administered alone, it is preferable to administer the compound as a pharmaceutical formulation (composition). The *hedgehog* antagonists according to the invention may be formulated for administration in any convenient way for use in human or veterinary medicine. In certain embodiments, the compound included in the pharmaceutical preparation may be

active itself, or may be a prodrug, e.g., capable of being converted to an active compound in a physiological setting.

Thus, another aspect of the present invention provides pharmaceutically acceptable compositions comprising a therapeutically effective amount of one or more of the compounds described above, formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. As described in detail below, the pharmaceutical compositions of the present invention may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes for application to the tongue; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment or spray applied to the skin; or (4) intravaginally or intrarectally, for example, as a pessary, cream or foam. However, in certain embodiments the subject compounds may be simply dissolved or suspended in sterile water. In certain embodiments, the pharmaceutical preparation is non-pyrogenic, i.e., does not elevate the body temperature of a patient.

The phrase "therapeutically effective amount" as used herein means that amount of a compound, material, or composition comprising a compound of the present invention which is effective for producing some desired therapeutic effect by overcoming a *hedgehog* gain-of-function phenotype in at least a sub-population of cells in an animal and thereby blocking the biological consequences of that pathway in the treated cells, at a reasonable benefit/risk ratio applicable to any medical treatment.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically acceptable carrier" as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid

filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject antagonists from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

As set out above, certain embodiments of the present *hedgehog* antagonists may contain a basic functional group, such as amino or alkylamino, and are, thus, capable of forming pharmaceutically acceptable salts with pharmaceutically acceptable acids. The term "pharmaceutically acceptable salts" in this respect, refers to the relatively non-toxic, inorganic and organic acid addition salts of compounds of the present invention. These salts can be prepared *in situ* during the final isolation and purification of the compounds of the invention, or by separately reacting a purified compound of the invention in its free base form with a suitable organic or inorganic acid, and isolating the salt thus formed. Representative salts include the hydrobromide, hydrochloride, sulfate, bisulfate, phosphate, nitrate, acetate, valerate, oleate, palmitate, stearate, laurate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, naphylate, mesylate, glucoheptonate, lactobionate, and laurylsulphonate salts and the like. (See, for example, Berge et al. (1977) "Pharmaceutical Salts", *J. Pharm. Sci.* 66:1-19)

The pharmaceutically acceptable salts of the subject compounds include the conventional nontoxic salts or quaternary ammonium salts of the compounds, e.g., from non-toxic organic or inorganic acids. For example, such conventional nontoxic salts include those derived from inorganic acids such as hydrochloride, hydrobromic, sulfuric, sulfamic, phosphoric, nitric, and the like; and the salts prepared from organic acids such as acetic, propionic, succinic, glycolic, stearic, lactic, malic, tartaric, citric, ascorbic, palmitic, maleic, hydroxymaleic, phenylacetic, glutamic, benzoic, salicylic, sulfanilic, 2-acetoxybenzoic, fumaric, toluenesulfonic, methanesulfonic, ethane disulfonic, oxalic, isothionic, and the like.

In other cases, the compounds of the present invention may contain one or more acidic functional groups and, thus, are capable of forming pharmaceutically acceptable salts with pharmaceutically acceptable bases. The term "pharmaceutically acceptable salts" in these instances refers to the relatively non-toxic, inorganic and organic base addition salts of compounds of the present invention. These salts can likewise be prepared *in situ* during the final isolation and purification of the compounds, or by separately reacting the purified compound in its free acid form with a suitable base, such as the hydroxide, carbonate or bicarbonate of a pharmaceutically acceptable metal cation, with ammonia, or with a pharmaceutically acceptable organic primary, secondary or tertiary amine. Representative alkali or alkaline earth salts include the lithium, sodium, potassium, calcium, magnesium, and aluminum salts and the like. Representative organic amines useful for the formation of base addition salts include ethylamine, diethylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine and the like. (See, for example, Berge et al., *supra*)

Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Examples of pharmaceutically acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl

palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Formulations of the present invention include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 1 per cent to about ninety-nine percent of active ingredient, preferably from about 5 per cent to about 70 per cent, most preferably from about 10 per cent to about 30 per cent.

Methods of preparing these formulations or compositions include the step of bringing into association a compound of the present invention with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a compound of the present invention with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Formulations of the invention suitable for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a compound of the present invention as an active ingredient. A compound of the present invention may also be administered as a bolus, electuary or paste.

In solid dosage forms of the invention for oral administration (capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more pharmaceutically acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

The tablets, and other solid dosage forms of the pharmaceutical compositions of the present invention, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release

profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. The active ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

Liquid dosage forms for oral administration of the compounds of the invention include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

It is known that sterols, such as cholesterol, will form complexes with cyclodextrins. Thus, in preferred embodiments, where the inhibitor is a steroidal alkaloid, it may be formulated with cyclodextrins, such as α -, β - and γ -cyclodextrin, dimethyl- β cyclodextrin and 2-hydroxypropyl- β -cyclodextrin.

Formulations of the pharmaceutical compositions of the invention for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing one or more compounds of the invention with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active *hedgehog* antagonist.

Formulations of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate.

Dosage forms for the topical or transdermal administration of a compound of this invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

The ointments, pastes, creams and gels may contain, in addition to an active compound of this invention, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to a compound of this invention, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Transdermal patches have the added advantage of providing controlled delivery of a compound of the present invention to the body. Such dosage forms can be made by dissolving or dispersing the *hedgehog* antagonists in the proper medium. Absorption enhancers can also be used to increase the flux of the *hedgehog* antagonists across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the compound in a polymer matrix or gel.

Ophthalmic formulations, eye ointments, powders, solutions and the like, are also contemplated as being within the scope of this invention.

Pharmaceutical compositions of this invention suitable for parenteral administration comprise one or more compounds of the invention in combination with one or more pharmaceutically acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form.

Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microencapsule matrices of the subject compounds in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue.

When the compounds of the present invention are administered as pharmaceuticals, to humans and animals, they can be given per se or as a pharmaceutical composition containing, for example, 0.1 to 99.5% (more preferably, 0.5 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

The addition of the active compound of the invention to animal feed is preferably accomplished by preparing an appropriate feed premix containing the active compound in an effective amount and incorporating the premix into the complete ration.

Alternatively, an intermediate concentrate or feed supplement containing the active ingredient can be blended into the feed. The way in which such feed premixes and complete rations can be prepared and administered are described in reference books (such as "Applied Animal Nutrition", W.H. Freedman and CO., San Francisco, U.S.A., 1969 or "Livestock Feeds and Feeding" O and B books, Corvallis, Ore., U.S.A., 1977).

VI. Synthetic Schemes and Identification of Active Antagonists

The subjects steroidal alkaloids, and congeners thereof, can be prepared readily by employing the cross-coupling technologies of Suzuki, Stille, and the like. These coupling reactions are carried out under relatively mild conditions and tolerate a wide range of "spectator" functionality.

a. Combinatorial Libraries

The compounds of the present invention, particularly libraries of variants having various representative classes of substituents, are amenable to combinatorial chemistry and other parallel synthesis schemes (see, for example, PCT WO 94/08051). The result is that large libraries of related compounds, e.g. a variegated library of compounds represented above, can be screened rapidly in high throughput assays in order to identify potential *hedgehog* antagonist lead compounds, as well as to refine the specificity, toxicity, and/or cytotoxic-kinetic profile of a lead compound. For instance, *ptc*, *hedgehog*, or *smoothened* bioactivity assays, such as may be developed using cells with either a *ptc* loss-of-function, *hedgehog* gain-of-function, or *smoothened* gain-of-function, can be used to screen a library of the subject compounds for those having agonist activity toward *ptc* or antagonist activity towards *hedgehog* or *smoothened*.

Simply for illustration, a combinatorial library for the purposes of the present invention is a mixture of chemically related compounds which may be screened together for a desired property. The preparation of many related compounds in a single reaction greatly reduces and simplifies the number of screening processes which need to be carried out. Screening for the appropriate physical properties can be done by conventional methods.

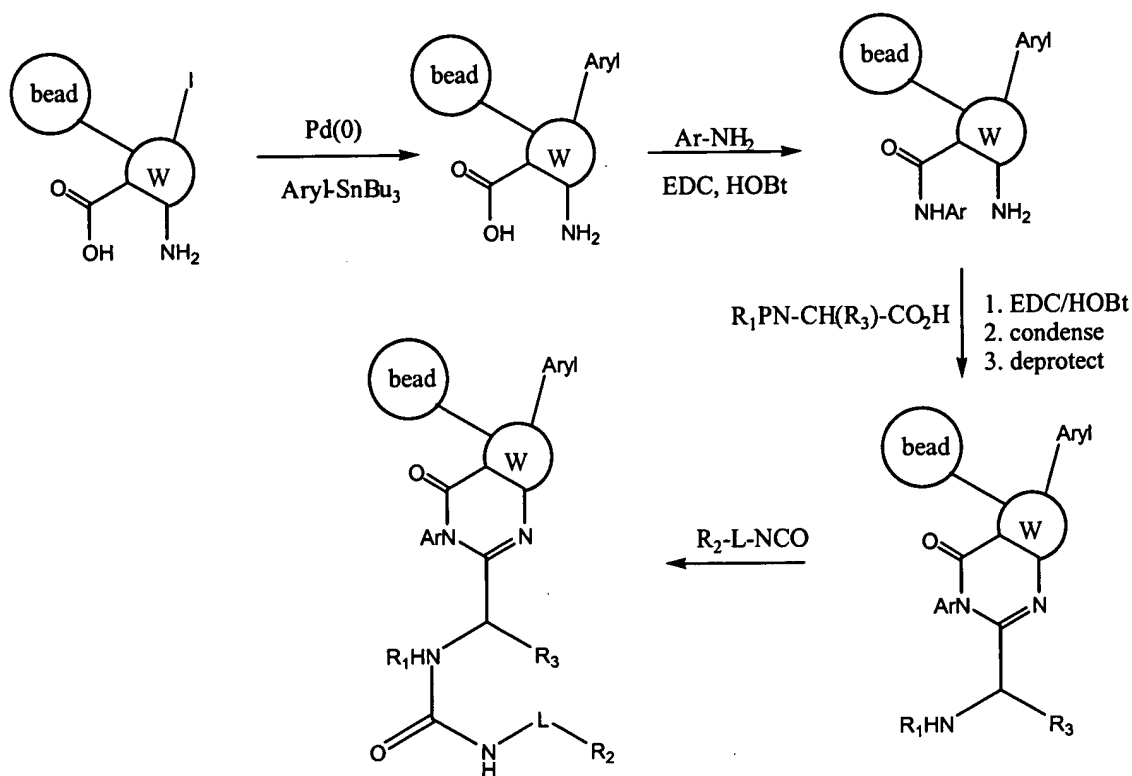
Diversity in the library can be created at a variety of different levels. For instance, the substrate aryl groups used in the combinatorial reactions can be diverse in terms of the core aryl moiety, e.g., a variegation in terms of the ring structure, and/or can be varied with respect to the other substituents.

A variety of techniques are available in the art for generating combinatorial libraries of small organic molecules such as the subject *hedgehog* antagonists. See, for example, Blondelle et al. (1995) Trends Anal. Chem. 14:83; the Affymax U.S. Patents 5,359,115 and 5,362,899; the Ellman U.S. Patent 5,288,514; the Still et al. PCT publication WO 94/08051; the ArQule U.S. Patents 5,736,412 and 5,712,171; Chen et al. (1994) JACS 116:2661; Kerr et al. (1993) JACS 115:252; PCT publications WO92/10092, WO93/09668 and WO91/07087; and the Lerner et al. PCT publication WO93/20242). Accordingly, a variety of libraries on the order of about 100 to 1,000,000

or more diversomers of the subject *hedgehog* antagonists can be synthesized and screened for particular activity or property.

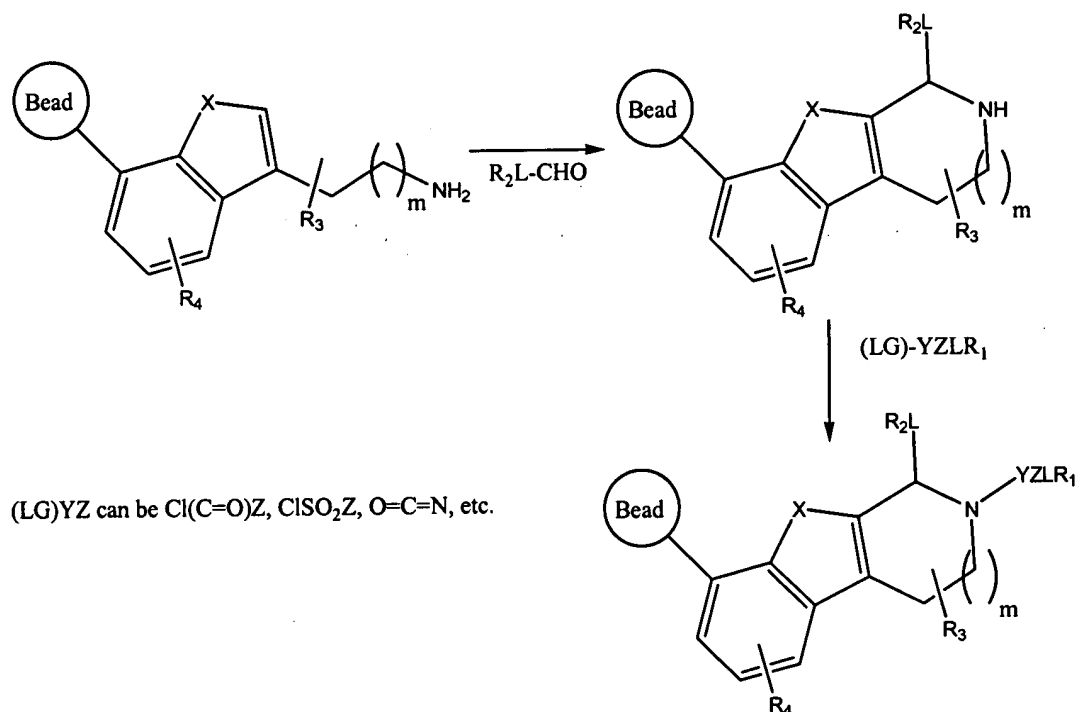
In an exemplary embodiment, a library of candidate *hedgehog* antagonists diversomers can be synthesized utilizing a scheme adapted to the techniques described in the Still et al. PCT publication WO 94/08051, e.g., being linked to a polymer bead by a hydrolyzable or photolyzable group, optionally located at one of the positions of the candidate antagonists or a substituent of a synthetic intermediate. According to the Still et al. technique, the library is synthesized on a set of beads, each bead including a set of tags identifying the particular diversomer on that bead. The bead library can then be "plated" with *ptc* loss-of-function, *hedgehog* gain-of-function, or *smoothed* gain-of-function cells for which an *hedgehog* antagonist is sought. The diversomers can be released from the bead, e.g. by hydrolysis.

The structures of the compounds useful in the present invention lend themselves readily to efficient synthesis. The nature of the structures, as generally described by formulas I and II, allows the assembly of such compounds using some combination of X, Y, and Z moieties, as set forth above. X and Z moieties are heteroatomic in nature, and thus allow the formation of diverse bonds to carbon. Y moieties, when present, take the form of electrophilic moieties, such as carbonyl and sulfonyl groups, and reagents are readily available for the attachment of such moieties to heteroatomic groups like X and Z. The vast majority of such reactions, including those depicted in Figures 11, 12, 15, and 16 are both extremely mild and extremely reliable, and are thus perfectly suited for combinatorial chemistry. The facile nature of such a combinatorial approach towards the generation of a library of test compounds is apparent in the exemplary scheme below (P = protecting group), wherein the various groups of a compound according to Formula II are linked combinatorially (e.g., using one of the methods described above), with combinatorial functionalization of the core ring system (e.g., W) bestowing additional diversity to the library. Even greater diversity may be attained by, for example, utilizing a range of electrophilic groups when appending a subunit, i.e., using a range of PO-Ar-L-C(O)Cl, PO-Ar-L-NCO, PO-Ar-L-SO₂Cl, etc. when appending the R₂ subunit.



Many variations on the above and related pathways permit the synthesis of widely diverse libraries of compounds which may be tested as inhibitors of *hedgehog* function.

Similarly, the structures of the compounds of Formulae III and IV lend themselves readily to efficient synthesis. The nature of the structures, as generally described by formulas III and IV, allows the assembly of such compounds using some combination of X, Y, and Z moieties, as set forth above. Attachment of the YZLR_1 fragment to the nitrogen of the heterocycle permits a wide range of versatile coupling reactions to be used, including reacting acid chlorides, sulfonyl chlorides, isocyanates, and others. The vast majority of such reactions, including those depicted in Figures 11, 12, 15, and 16 are both extremely mild and extremely reliable, and are thus perfectly suited for combinatorial chemistry. The facile nature of such a combinatorial approach towards the generation of a library of test compounds is apparent in the exemplary scheme, wherein the various groups of a subject compound are linked combinatorially (e.g., using one of the methods described above). Even greater diversity may be attained by, for example, employing a ketone instead of an aldehyde in the cyclization step, employing a range of different heterocycles (other than, e.g., indole), alkylating the ring nitrogen with a functionalized L group prior to appending a YZLR_1 , alkylating the ring nitrogen with a protected LY group, prior to appending ZLR_1 , etc.



Many variations on the above and related pathways permit the synthesis of widely diverse libraries of compounds which may be tested as inhibitors of *hedgehog* function.

b. Screening Assays

There are a variety of assays available for determining the ability of a compound to agonize *ptc* function or antagonize *smoothened* or *hedgehog* function, many of which can be disposed in high-throughput formats. In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Thus, libraries of synthetic and natural products can be sampled for other compounds which are *hedgehog* antagonists.

In addition to cell-free assays, test compounds can also be tested in cell-based assays. In one embodiment, cell which have a *ptc* loss-of-function, *hedgehog* gain-of-function, or *smoothened* gain-of-function phenotype can be contacted with a test agent of interest, with the assay scoring for, e.g., inhibition of proliferation of the cell in the presence of the test agent.

A number of gene products have been implicated in *patched*-mediated signal transduction, including *patched*, transcription factors of the *cubitus interruptus* (ci) family, the serine/threonine kinase *fused* (fu) and the gene products of *costal-2*, *smoothened* and *suppressor of fused*.

The induction of cells by hedgehog proteins sets in motion a cascade involving the activation and inhibition of downstream effectors, the ultimate consequence of which is, in some instances, a detectable change in the transcription or translation of a gene. Potential transcriptional targets of *hedgehog*-mediated signaling are the *patched* gene (Hidalgo and Ingham, 1990 Development 110, 291-301; Marigo et al., 1996) and the vertebrate homologs of the drosophila cubitus interruptus gene, the *GLI* genes (Hui et al. (1994) Dev Biol 162:402-413). *Patched* gene expression has been shown to be induced in cells of the limb bud and the neural plate that are responsive to *Shh*. (Marigo et al. (1996) PNAS 93:9346-51; Marigo et al. (1996) Development 122:1225-1233). The *Gli* genes encode putative transcription factors having zinc finger DNA binding domains (Orenic et al. (1990) Genes & Dev 4:1053-1067; Kinzler et al. (1990) Mol Cell Biol 10:634-642). Transcription of the *Gli* gene has been reported to be upregulated in response to *hedgehog* in limb buds, while transcription of the *Gli3* gene is downregulated in response to *hedgehog* induction (Marigo et al. (1996) Development 122:1225-1233). By selecting transcriptional regulatory sequences from such target genes, e.g., from *patched* or *Gli* genes, that are responsible for the up- or down-regulation of these genes in response to *hedgehog* signalling, and operatively linking such promoters to a reporter gene, one can derive a transcription based assay which is sensitive to the ability of a specific test compound to modify *hedgehog*-mediated signalling pathways. Expression of the reporter gene, thus, provides a valuable screening tool for the development of compounds that act as antagonists of *hedgehog*.

Reporter gene based assays of this invention measure the end stage of the above described cascade of events, e.g., transcriptional modulation. Accordingly, in practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on *ptc* loss-of-function, *hedgehog* gain-of-function, *smoothened* gain-of-function, or stimulation by SHH itself. The amount of

transcription from the reporter gene may be measured using any method known to those of skill in the art to be suitable. For example, mRNA expression from the reporter gene may be detected using RNase protection or RNA-based PCR, or the protein product of the reporter gene may be identified by a characteristic stain or an intrinsic biological activity. The amount of expression from the reporter gene is then compared to the amount of expression in either the same cell in the absence of the test compound or it may be compared with the amount of transcription in a substantially identical cell that lacks the target receptor protein. Any statistically or otherwise significant decrease in the amount of transcription indicates that the test compound has in some manner agonized the normal *ptc* signal (or antagonized the gain-of-function *hedgehog* or *smoothened* signal), e.g., the test compound is a potential *hedgehog* antagonist.

Exemplification

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Lead Compound Discovery/ High-throughput Screening Assay

Compounds to be tested are dissolved in DMSO to a concentration of 10 mM, and stored at -20 °C. To activate the *Hedgehog* pathway in the assay cells, an octylated (lipid-modified) form of the N-terminal fragment of the *Sonic Hedgehog* protein (OCT-SHH) is used. This N-terminal SHH fragment is produced bacterially. The octylation involves reaction of the N-terminal fragment, via its amino-terminal cysteine moiety, with octyl maleimide. This modified form, like others described in Pepinsky et al., *J. Biol. Chem.* 1998, 273, 14037-45, exhibits higher specific potency than the bacterially derived unmodified fragment in several cell-based assays of hedgehog signalling.

Compounds may be tested in the "Gli-Luc" assay below, using the cell line 10T(s12), wherein the cells contain a Hedgehog-responsive reporter construct utilizing Luciferase as the reporter gene. In this way, *Hedgehog* pathway signaling activity can be measured via the Gli-Luc response.

10t1/2(s12) cells are plated in a 96-well micro-titer plate (MTP) at 20,000 cells/well in full medium [DMEM with 10% FBS]. Then plates are placed in the incubator for incubation overnight (O/N), at 37 °C and 5% CO₂. After 24 h, the medium is replaced with Luciferase-assay medium (DMEM with 0.5% FBS). Compounds are thawed and diluted in assay medium at 3:1000 (about 300-fold) resulting in a starting concentration of about 30 µM.

Subsequently, 150 µl of each 30 µM sample is added to the first wells (in triplicate). The MTP samples are then diluted at 3-fold dilutions to a total of seven wells, ultimately resulting in a regiment of seven dilutions in triplicate, for each compound. Next, the protein ligand OCT-SHH is diluted in Luciferase-assay medium and added to each well at a final concentration of 0.3 µg/ml. Plates are then returned to the incubator for further incubation O/N, at 37 °C and 5% CO₂. After about 24 h, plates are removed from the incubator and the medium is aspirated/discarded. Wells are washed once with assay buffer [PBS + 1 mM Mg²⁺ and 1 mM Ca²⁺]. Then 50 µl of assay buffer is added to each well. The Luciferase assay reagent is prepared as described by the vendor (LucLite kit from Packard), and 50 µl is added to each well. Plates are incubated at room temperature (RT) for about 30 minutes after which the signals are read, again at RT, on a Topcount (Packard).

Compounds identified in this assay are depicted in Figure 32. The discovery of these Shh-induced Gli-transcription activity inhibitors exemplifies the utility of the claims in this patent. Activities for these compounds are presented in Table 1 below.

Table 1

Compound	IC ₅₀	Compound	IC ₅₀
31	<10 µM	55	<5 µM
32	<5 µM	56	<10 µM
34	<5 µM	57	<10 µM
11	<5 µM	58	<5 µM

36	<5 μ M	59	<5 μ M
38	<5 μ M	60	<5 μ M
39	<5 μ M	61	<1 μ M
40	<10 μ M	62	<1 μ M
41	<10 μ M	63	<10 μ M
42	<5 μ M	64	<10 μ M
43	<10 μ M	65	<10 μ M
44	<1 μ M	66	<10 μ M
45	<5 μ M	67	<5 μ M
46	<0.5 μ M	68	<1 μ M
47	<5 μ M	69	<0.5 μ M
48	<0.5 μ M	5	<0.1 μ M
49	<1 μ M	71	<10 μ M
50	<1 μ M	6	<0.5 μ M
51	<5 μ M	73	<5 μ M
52	<1 μ M	74	<5 μ M
53	<1 μ M	75	<5 μ M
54	<5 μ M		

Additional data is presented in Figure 33. Mouse 456 is a *Ptc*-knockout heterozygote that received UV irradiation for 6 months. The mouse developed many small BCC lesions, which were blue after X-gal staining. The mouse was sacrificed and the skin was excised with a 2 mm skin punch. Those skin punches were then cultured for 6 days. Comparing to vehicle (DMSO), compound 11 can decrease the number and size of BCC lesions (blue spots in the picture). The effect was more obvious when 10 μ M of 11 was added to the culture medium instead of 5 μ M of 11. In short, this experiment suggests that 11 is able to inhibit murine BCC lesions in mouse #456.

In yet another experiment, E12.5 old *ptc-1 (d11) lacZ* lungs were harvested and transgenic embryos identified by *lacZ* detection using tails. Lung explants were grown submerged in mouse explant medium (DMEM based, additives optimized for the culture of mouse lungs) for 48 hrs, fixed in *lacZ* fixative, rinsed and stained for *lacZ* O/N at 37 °C. Control tissue was untreated, while test tissue was treated with 11. Results are depicted in Figure 34: (A) Untreated control. Strong *lacZ* expression can be observed in distal and proximal mesenchyme. (B) Treatment with 11 leads to significantly decreased reporter gene expression, as evidenced especially by the weak signal surrounding the distal branching tips of the growing lung epithelium.

Ptc-null Assay

Methods

Ptc-null cells were cultured for 3 days in the presence of vehicle; jervine, a known Patched pathway antagonist (i) used here as a positive control; or 1 μ M of a test compound. Total ribonucleic acid (RNA) was isolated from the cells and used for reverse transcriptase-polymerase chain reaction (RT-PCR). Specific primers for the detection of mouse *gli-1* mRNA were used in the PCR, and the actin gene was used to demonstrate that equivalent amounts of mRNA samples were compared in the experiment. The *gli-1* and actin mRNA samples were then loaded on 1.5% agarose gel and were detected by staining with ethidium bromide. The same samples were analyzed by the quantitative real-time polymerase chain reaction method to quantify the levels of *gli-1* mRNA.

Compared with vehicle, jervine significantly decreased the expression of *gli-1* mRNA in *ptc*-null cells. The levels of actin mRNA were equivalent in all conditions, indicating that equal quantities of RNA were analyzed in the experiment. However, no significant decrease in the expression of *gli-1* was observed in the presence of compounds 11, 47, 48, 49, 77, 78, 80, and 81.

Preparation of compounds of the present invention

a. Illustrative synthetic schemes

Exemplary synthesis schemes for generating *hedgehog* antagonists useful in the methods and compositions of the present invention are shown in Figures 1-31.

The reaction conditions in the illustrated schemes of Figure 1-31 are as follows:

- 1) R_1CH_2CN , $NaNH_2$, toluene
(Arzneim-Forsch, 1990, 40, 11, 1242)
- 2) H_2SO_4 , H_2O , reflux
(Arzneim-Forsch, 1990, 40, 11, 1242)
- 3) H_2SO_4 , EtOH, reflux

(Arzneim-Forsch, 1990, 40, 11, 1242)

4) NaOH, EtOH, reflux

5) (Boc)₂O, 2M NaOH, THF

6) LiHDMS, R₁X, THF

(Merck Patent Applic # WO 96/06609)

7) Pd-C, H₂, MeOH

8) t-BuONO, CuBr, HBr, H₂O

(J. Org. Chem. 1977, 42, 2426)

9) ArB(OH)₂, Pd(PPh₃)₄, Dioxane

(J. Med. Chem. 1996, 39, 217-223)

10) R₁₂(H)C=CR₁₃R₁₄, Pd(OAc)₂, Et₃N, DMF

(Org. React. 1982, 27, 345)

11) Tf₂O, THF

(J. Am. Chem. Soc. 1987, 109, 5478-5486)

12) ArSnBu₃, Pd(PPh₃)₄, Dioxane

(J. Am. Chem. Soc. 1987, 109, 5478-5486)

13) KMnO₄, Py, H₂O

(J. Med. Chem. 1996, 39, 217-223)

14) NaOR₁, THF

15) NaSR₁, THF

16) HNR₁R₁₃, THF

17) HONO, NaBF₄

(Adv. Fluorine Chem. 1965, 4, 1-30)

18) Pd(OAc)₂, NaH, DPPF, PhCH₃, R₁OH

(J. Org. Chem. 1997, 62, 5413-5418)

19) i. R₁X, Et₃N, CH₂Cl₂, ii. R₁₃X

20) SOCl₂, cat DMF

21) CH₂N₂, Et₂O

22) Ag₂O, Na₂CO₃, Na₂S₂O₃, H₂O

(Tetrahedron Lett. 1979, 2667)

23) AgO_2CPh , Et_3N , MeOH

(Org. Syn., 1970, 50, 77; J. Am. Chem. Soc. 1987, 109, 5432)

24) LiOH , THF-MeOH

25) $(\text{EtO})_2\text{P}(\text{O})\text{CH}_2\text{CO}_2\text{R}$, BuLi , THF

26) $\text{MeO}_2\text{CCH}(\text{Br})=\text{P}(\text{Ph})_3$, benzene

27) KOH or KOtBu

28) Base, $\text{X}(\text{CH}_2)_n\text{CO}_2\text{R}$

29) DPPA , Et_3N , toluene

(Synthesis 1985, 220)

30) HONO , H_2O

31) SO_2 , CuCl , HCl , H_2O

(Synthesis 1969, 1-10, 6)

32) Lawesson's reagent, toluene

(Tetrahedron Asym. 1996, 7, 12, 3553)

33) R_2M , solvent

34) 30% H_2O_2 , glacial $\text{CH}_3\text{CO}_2\text{H}$

(Helv. Chim. Acta. 1968, 349, 323)

35) triphosgene, CH_2Cl_2

(Tetrahedron Lett., 1996, 37, 8589)

36) i. $(\text{EtO})_2\text{P}(\text{O})\text{CHLiSO}_2\text{Oi-Pr}$, THF , ii. NaI

37) $\text{Ph}_3\text{PCH}_3\text{I}$, $\text{NaCH}_2\text{S}(\text{O})\text{CH}_3$, DMSO

(Synthesis 1987, 498)

38) Br_2 , CHCl_3 or other solvent

(Synthesis 1987, 498)

39) BuLi , Bu_3SnCl

40) ClSO_2OTMS , CCl_4

(Chem. Ber. 1995, 128, 575-580)

41) MeOH-HCl , reflux

- 42) LAH, Et₂O or LiBH₄, EtOH or BH₃-THF
(Tetrahedron Lett., 1996, 37, 8589)
- 43) MsCl, Et₃N, CH₂Cl₂
(Tetrahedron Lett., 1996, 37, 8589)
- 44) Na₂SO₃, H₂O
(Tetrahedron Lett., 1996, 37, 8589)
- 45) R₂R₄NH, Et₃N, CH₂Cl₂
- 46) R₂M, solvent
- 47) CH₃NH(OCH₃), EDC, HOBt, DIEA, CH₂Cl₂ or DMF
(Tetrahedron Lett., 1981, 22, 3815)
- 48) MeLi, THF
- 49) mCPBA, CH₂Cl₂
- 50) HONO, Cu₂O, Cu(NO₃)₂, H₂O
(J. Org. Chem. 1977, 42, 2053)
- 51) R₁M, solvent
- 52) HONO, NaS(S)COEt, H₂O
(Org. Synth. 1947, 27, 81)
- 53) HSR₂ or HSR₄, CH₂Cl₂
- 54) i-BuOC(O)Cl, Et₃N, NH₃, THF
- 55) R₂R₄NH, CH₂Cl₂, NaBH(OAc)₃
- 56) R₂R₄NH, MeOH/CH₃CO₂H, NaBH₃CN
- 57) R₂OH, EDC, HOBt, DIEA, CH₂Cl₂ or DMF
- 58) R₂OH, HBTU, HOBt, DIEA, CH₂Cl₂ or DMF
- 59) R₂R₄NH, EDC, HOBt, DIEA, CH₂Cl₂ or DMF
- 60) R₂R₄NH, HBTU, HOBt, DIEA, CH₂Cl₂ or DMF
- 61) POCl₃, Py, CH₂Cl₂
- 62) R₂R₄NCO, solvent
- 63) R₂OC(O)Cl, Et₃N, solvent
- 64) R₂CO₂H, EDC or HBTU, HOBt, DIEA, CH₂Cl₂ or DMF

- 65) R_2X , Et_3N , solvent
- 66) $(CH_3S)_2C=N(CN)$, DMF, EtOH
(J. Med. Chem. 1994, 37, 57-66)
- 67) R_2SO_2Cl , Et_3N , CH_2Cl_2
- 68) R_2 - or R_3 - or R_4CHO , $MeOH/CH_3CO_2H$, $NaBH_3CN$
(Synthesis 1975, 135-146)
- 69) Boc(Tr)-D or L-CysOH, HBTU, HOBt, DIEA, CH_2Cl_2 or DMF
- 70) Boc(Tr)-D or L-CysH, $NaBH_3CN$, $MeOH/CH_3CO_2H$
(Synthesis 1975, 135-146)
- 71) S-Tr-N-Boc cysteinyl, $ClCH_2CH_2Cl$ or THF, $NaBH(OAc)_3$
(J. Org. Chem. 1996, 61, 3849-3862)
- 72) TFA, CH_2Cl_2 , Et_3SiH or (3:1:1) thioanisole/ethanedithiol/DMS
- 73) TFA, CH_2Cl_2
- 74) DPPA, Et_3N , toluene, $HOCH_2CH_2SiCH_3$
(Tetrahedron Lett. 1984, 25, 3515)
- 75) TBAF, THF
- 76) Base, TrSH or BnSH
- 77) Base, R_2X or R_4X
- 78) R_3NH_2 , $MeOH/CH_3CO_2H$, $NaBH_3CN$
- 79) N_2H_4 , KOH
- 80) $Pd_2(dba)_3$, $P(o-tol)_3$, RNH_2 , $NaOtBu$, Dioxane, R_1NH_2
(Tetrahedron Lett. 1996, 37, 7181-7184).
- 81) Cyanamide.
- 82) Fmoc-Cl, sodium bicarbonate.
- 83) BnCOCl, sodium carbonate.
- 84) AllylOCOCl, pyridine.
- 85) Benzyl bromide, base.
- 86) Oxalyl chloride, DMSO.
- 87) $RCONH_2$.
- 88) Carbonyldiimidazole, neutral solvents (e.g., DCM, DMF, THF, toluene).

- 89) Thiocarbonyldiimidazole, neutral solvents (e.g., DCM, DMF, THF, toluene).
- 90) Cyanogen bromide, neutral solvents (e.g., DCM, DMF, THF, toluene).
- 91) RCOCl, Triethylamine
- 92) RNHNH₂, EDC.
- 93) RO₂CCOCl, Et₃N, DCM.
- 94) MsOH, Pyridine (J. Het. Chem., 1980, 607.)
- 95) Base, neutral solvents (e.g., DCM, toluene, THF).
- 96) H₂NOR, EDC.
- 97) RCSNH₂.
- 98) RCOCHBrR, neutral solvents (e.g., DCM, DMF, THF, toluene), (Org. Proc. Prep. Intl., 1992, 24, 127).
- 99) CH₂N₂, HCl. (Synthesis, 1993, 197).
- 100) NH₂NHR, neutral solvents (e.g., DCM, DMF, THF, toluene).
- 101) RSO₂Cl, DMAP. (Tetrahedron Lett., 1993, 34, 2749).
- 102) Et₃N, RX. (J. Org. Chem., 1990, 55, 6037).
- 103) NOCl or Cl₂ (J. Org. Chem., 1990, 55, 3916).
- 104) H₂NOH, neutral solvents (e.g., DCM, DMF, THF, toluene).
- 105) RCCR, neutral solvents (DCM, THF, Toluene).
- 106) RCHCHR, neutral solvents (DCM, THF, Toluene).
- 107) H₂NOH, HCl.
- 108) Thiocarbonyldiimidazole, SiO₂ or BF₃OEt₂. (J. Med. Chem., 1996, 39, 5228).
- 109) Thiocarbonyldiimidazole, DBU or DBN. (J. Med. Chem., 1996, 39, 5228).
- 110) HNO₂, HCl.
- 111) ClCH₂CO₂Et (Org. Reactions, 1959, 10, 143).
- 112) Morpholine enamine (Eur. J. Med. Chem., 1982, 17, 27).
- 113) RCOCHR'CN
- 114) RCOCHR'CO₂Et
- 115) Na₂SO₃
- 116) H₂NCHR'CO₂Et

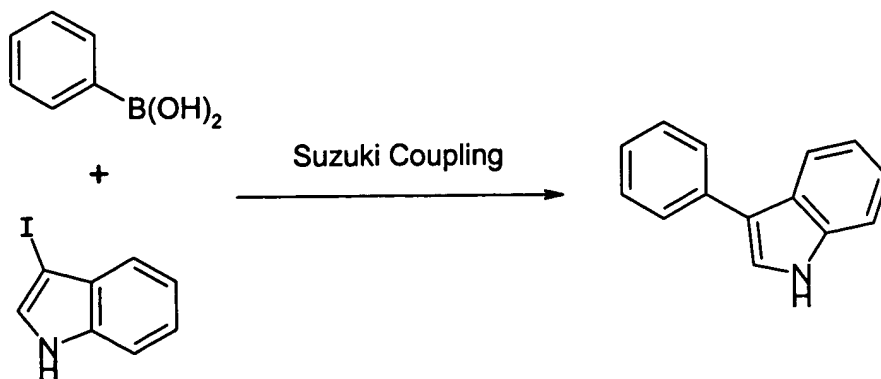
- 117) EtO₂CCHRNCO
- 118) RCNHNH₂.
- 119) RCOCO₂H, (J. Med. Chem., 1995, 38, 3741).
- 120) RCHO, KOAc.
- 121) 2-Fluoronitrobenzene.
- 122) SnCl₂, EtOH, DMF.
- 123) RCHO, NaBH₃CN, HOAc.
- 124) NH₃, MeOH.
- 125) 2,4,6-Me₃PhSO₂NH₂.
- 126) Et₂NH, CH₂Cl₂
- 127) MeOC(O)Cl, Et₃N, CH₂Cl₂
- 128) R₂NH₂, EDC, HOBT, Et₃N, CH₂Cl₂
- 129) DBU, PhCH₃
- 130) BocNHCH(CH₂STr)CH₂NH₂, EDC, HOBT, Et₃N, CH₂Cl₂
- 131) R₂NHCH₂CO₂Me, HBTU, HOBT, Et₃N, CH₂Cl₂
- 132) BocNHCH(CH₂STr)CH₂OMs, LiHMDS, THF
- 133) R₂NHCH₂CO₂Me, NaBH(OAc)₃, ClCH₂CH₂Cl or THF
- 134) R₂NHCH₂CH(OEt)₂, HBTU, HOBT, Et₃N, CH₂Cl₂
- 135) NaBH(OAc)₃, ClCH₂CH₂Cl or THF, AcOH.
- 136) Piperidine, DMF.
- 137) Pd(Ph₃P)₄, Bu₃SnH.
- 138) RCO₂H, EDC, HOBT, Et₃N, DCM.
- 139) RNH₂, neutral solvents.
- 140) RCHO, NaBH₃CN, HOAc.
- 141) RNCO, solvent.
- 142) RCO₂H, EDC or HBTU, HOBT, DIEA, CH₂Cl₂ or DMF.
- 143) RCOCl, Triethylamine
- 144) RSO₂Cl, Et₃N, CH₂Cl₂.
- 145) SnCl₂, EtOH, DMF.

- 146) RNH_2 , EDC, HOBt, DIEA, CH_2Cl_2 or DMF.
- 147) Dibromoethane, Et_3N , CH_2Cl_2
- 148) Oxalyl chloride, neutral solvents.
- 149) LiOH , THF-MeOH.
- 150) Carbonyldiimidazole, neutral solvents (e.g., DCM, DMF, THF, toluene).
- 151) RNH_2 , Et_3N , CH_2Cl_2 .
- 152) Base, RX.
- 153) DBU, PhCH_3
- 154) DPPA, Et_3N , toluene (Synthesis 1985, 220)
- 155) SOCl_2 , cat DMF.
- 156) ArH , Lewis Acid (AlCl_3 , SnCl_4 , TiCl_4), CH_2Cl_2 .
- 157) $\text{H}_2\text{NCHRCO}_2\text{Et}$, neutral solvents.
- 158) $\text{BocHNCHRCO}_2\text{H}$, EDC OR HBTU, HOBt, DIEA, CH_2Cl_2 or DMF.
- 159) TFA, CH_2Cl_2 .

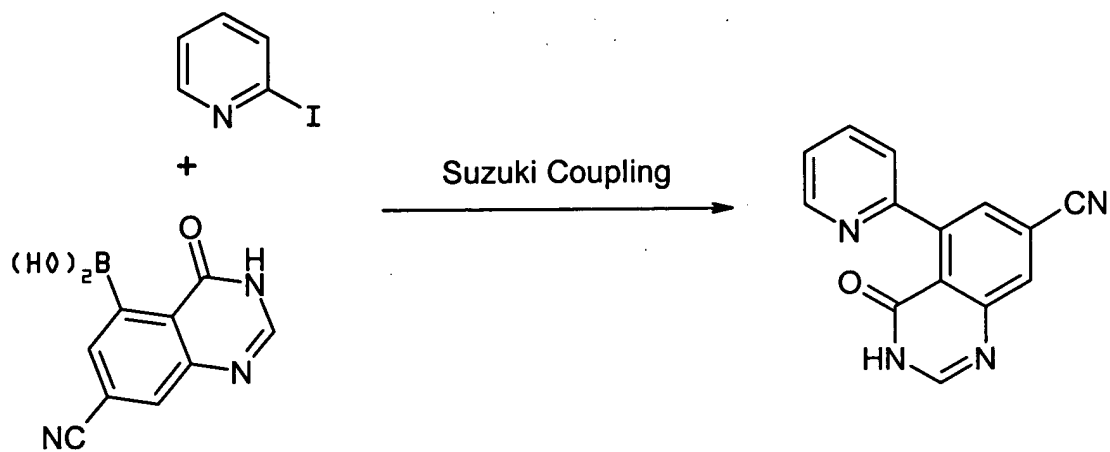
b. Illustrative preparation of aryl subunits

Ary subunits may be functionalized using a wide variety of reactions known to those in the art. The chemistry of aromatic and heteroaromatic rings is rich, and only a sampling of useful reactions can be presented here. A number of illustrative examples are shown below.

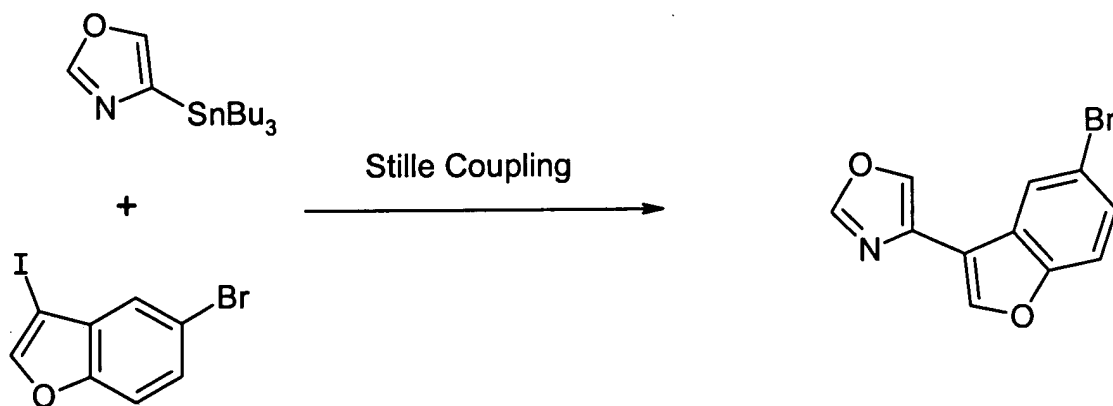
Suzuki Coupling No. 1:



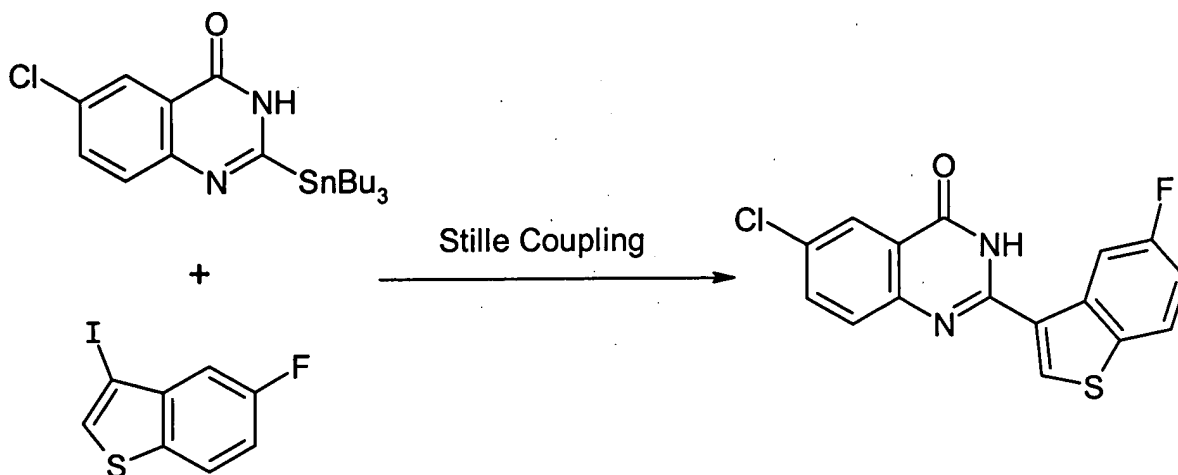
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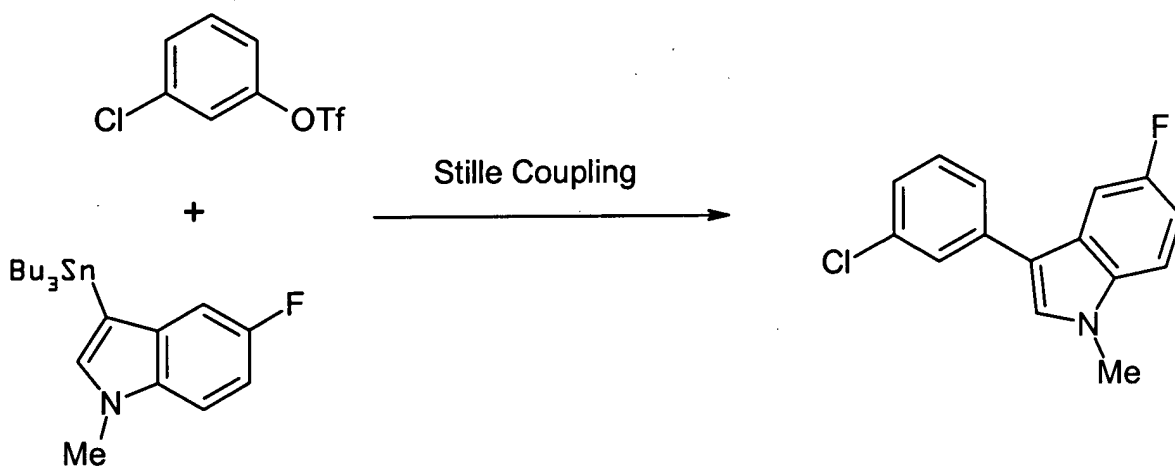
Stille Coupling No. 1:



Stille Coupling No. 2



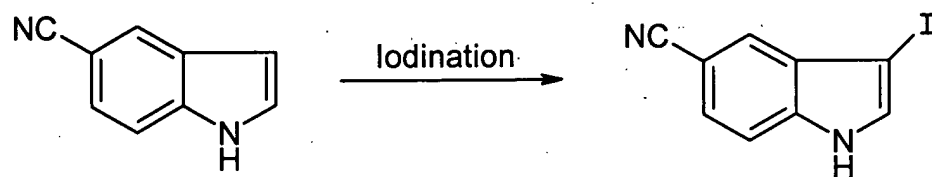
Stille Coupling No. 3



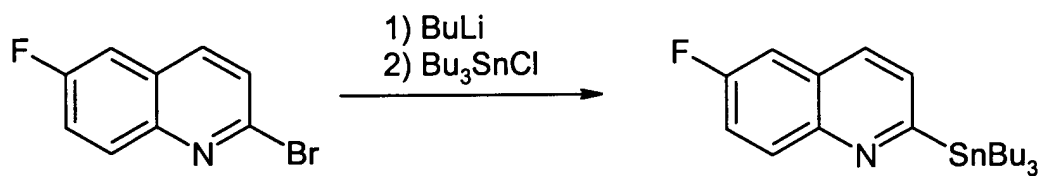
c. Illustrative preparation of coupling substrates

Members of the general classes of coupling substrates outlined above -- arylstannanes, arylboronic acids, aryl triflates and aryl halides -- are available from the parent heterocycles. In general, the transformations required to prepare a coupling substrate are reliable and amenable to scale-up. Illustrative examples are shown below.

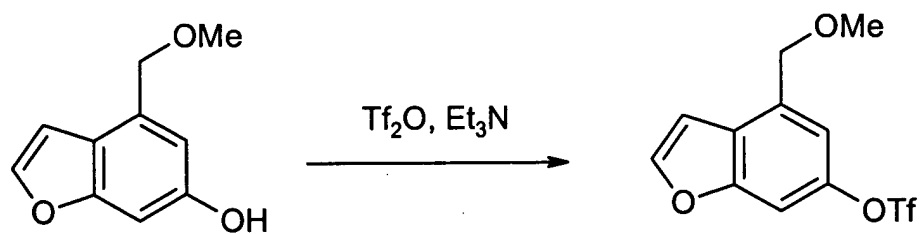
Preparation of an Aryl Iodide



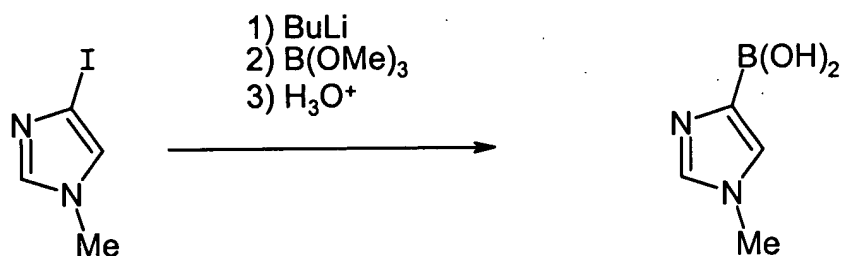
Preparation of an Aryl Stannane



Preparation of an Aryl Triflate



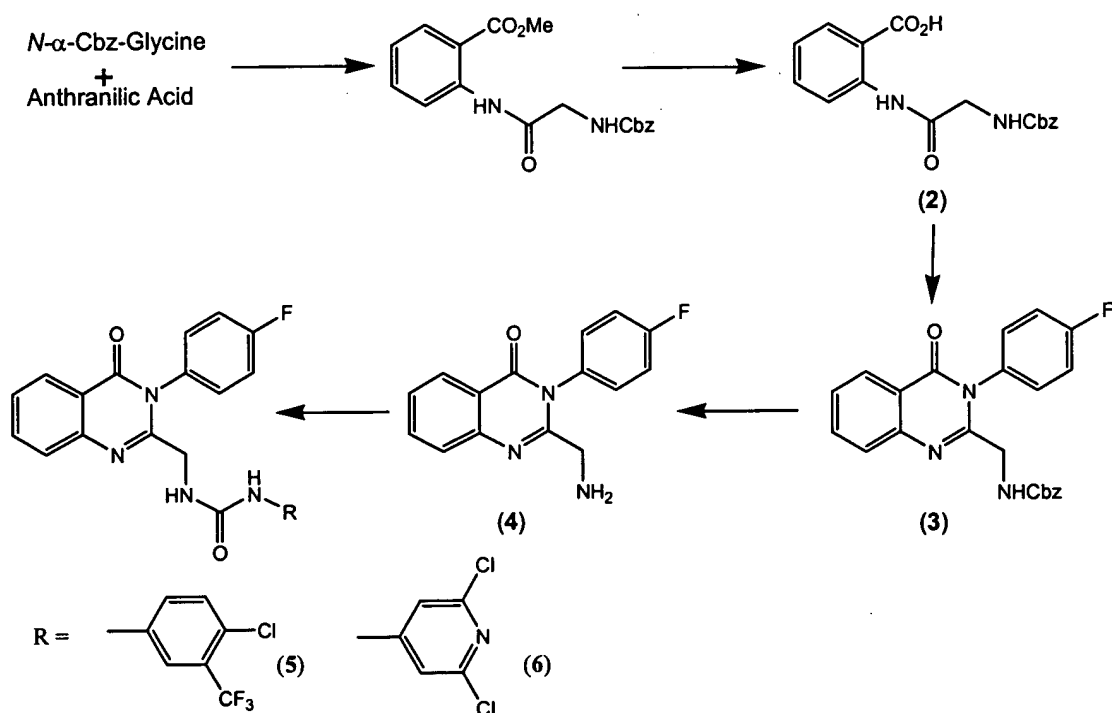
Preparation of Aryl Boronic Acid



d. Exemplary Procedures for Preparing Subject Compounds

Numbering of compounds within this section is independent of the compound numbering elsewhere in the application, and corresponds only to the numbering employed in the scheme preceding each set of experimental procedures.

Route 1



2-(2-Benzyloxycarbonylamino-acetylamino)-benzoic acid methyl ester (1)

To a solution of *N*- α -Cbz-glycine (2.0 g, 9.56 mmol) in tetrahydrofuran (20 mL) was added 1,1'-carbonyldiimidazole (1.64 g, 10.13 mmol), followed 2 h later by methyl anthranilate (1.28 g, 8.47 mmol). After stirring overnight the tetrahydrofuran was

removed in *vacuo* and the residue was redissolved in ethyl acetate, washed with 10% aqueous hydrochloric acid (2x), saturated aqueous sodium hydrogen carbonate (2x), dried (MgSO₄) and concentrated to give an oily residue which was subjected to silica-gel column chromatography [eluent: Ethyl acetate:Hexane, 30:70, v/v -> Ethyl acetate] to give the *title* benzoate (1) (0.31 g, 11%) as a white solid:

δ (360 MHz; CDCl₃) 3.89 (s, 3H), 4.10 (d, 2H), 5.19 (s, 2H), 7.11 (t, 1H), 7.31-7.41 (m, 5H), 7.56 (t, 1H), 8.03 (d, 1H), 8.69 (d, 1H) and 11.54 (s, 1H).

2-(2-Benzoyloxycarbonylamino-acetylamino)-benzoic acid (2)

Lithium hydroxide (55 mg, 1.32 mmol) in water (0.8 mL), was added to a solution of the benzoate (1) (300 mg, 0.88 mmol) in dioxane (4 mL). After stirring overnight, the reaction mixture was concentrated at 40 °C to give a viscous oil, which was treated with 10% aqueous hydrochloric acid (3 mL), with the resulting emulsion formed being extracted with ethyl acetate (4x). The organic phases were combined, dried (MgSO₄) and concentrated to give the *title* benzoic acid (2) quantitatively as a white solid:

δ (360 MHz; DMSO) 3.84 (d, 2H), 5.12 (s, 2H), 7.21 (t, 1H), 7.35-7.44 (m, 5H), 7.65 (t, 1H), 8.04 (t, 1H), 8.65 (d, 1H) and 11.74 (s, 1H).

[3-(4-Fluoro-phenyl)-4-oxo-3,4-dihydro-quinazolin-2-ylmethyl]-carbamic acid benzyl ester (3)

To a solution of the benzoic acid (2) (180 mg, 0.55 mmol) in tetrahydrofuran (6 mL) was added 1,1'-carbonyldiimidazole (98 mg, 0.60 mmol). After a period of 2 h, 4-fluoroaniline (61 mg, 0.55 mmol) in tetrahydrofuran (1.5 mL) was added and the mixture was stirred at 75 °C overnight. The tetrahydrofuran was removed in *vacuo* and the residue was redissolved in ethyl acetate, washed with 10% aqueous hydrochloric acid, saturated aqueous sodium hydrogen carbonate, dried (MgSO₄) and concentrated to give an oily residue which was subjected to silica-gel column chromatography [eluent: Ethyl acetate:Hexane, 20:80, v/v -> 60:40, v/v] to give the *title* quinazolinone (3) (0.11 g, 50%) as a white solid:

δ (360 MHz; CDCl₃) 4.01 (d, 2H), 5.13 (s, 2H), 6.27 (s, 1H), 7.31-7.39 (m, 9H), 7.52 (t, 1H), 7.71 (d, 1H), 7.81 (t, 1H) and 8.28 (d, 1H).

2-Aminomethyl-3-(4-Fluoro-phenyl)-3H-quinazolin-4-one (4)

A stirring mixture of the quinazolinone (3) (63 mg, 0.16 mmol), 10% palladium on activated carbon (18 mg) and methanol (4.4 mL) was evacuated using an aspirator pump and filled with hydrogen. Once the starting material had been consumed as monitored by TLC analysis, the mixture was filtered through a Celite pad which was washed with methanol (2x) and concentrated to give the *title* amine (4) (40 mg, 93%) as a yellow solid:

δ (360 MHz; CDCl₃) 3.50 (s, 2H), 7.25 (apparent d, 4H), 7.50 (t, 1H), 7.75-7.82 (m, 2H) and 8.28 (d, 1H).

1-(4-Chloro-3-trifluoromethyl-phenyl)-3-[3-(4-fluoro-phenyl)-4-oxo-3,4-dihydro-quinazolin-2-ylmethyl]-urea (5)

To a mixture of the amine (4) (14 mg, 0.05 mmol) in chloroform (0.5 mL), was added 3-trifluoromethylphenyl isocyanate (10 mg, 0.05 mmol). After stirring overnight the solvent was removed in *vacuo*, triturated with hexanes, filtered and dried to give the *title* urea (5) (24 mg, 98%) as a white solid:

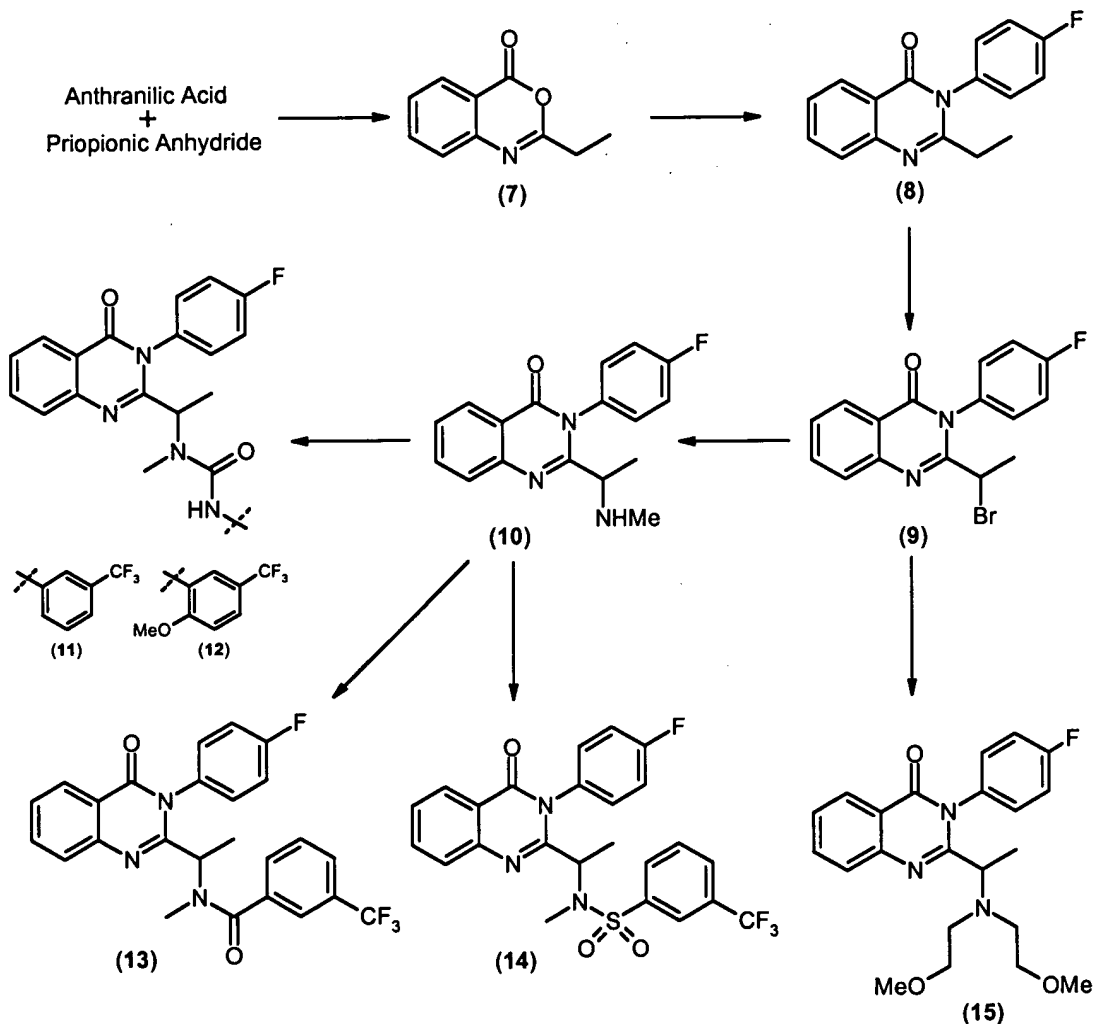
δ (360 MHz; CDCl₃) 4.08 (s, 2H), 6.52 (s, 1H), 7.16-7.38 (m, 4H), 7.48-7.53 (m, 2H), 7.59 (d, 1H), 7.66 (s 1H), 7.75 (t, 1H), 7.81 (s 1H) and 8.29 (d, 1H).

1-(2,6-Dichloro-pyridin-4-yl)-3-[3-(4-fluoro-phenyl)-4-oxo-3,4-dihydro-quinazolin-2-ylmethyl]-urea (6)

To a mixture of the amine (4) (14 mg, 0.05 mmol) in chloroform (0.5 mL), was added 2,6-dichloropyridyl isocyanate (11 mg, 0.05 mmol). After stirring overnight the solvent was removed in *vacuo*, triturated with hexanes, filtered and dried to give the *title* urea (6) (22 mg, 96%) as a white solid:

δ (360 MHz; CDCl₃) 4.09 (d, 2H), 6.41 (br s, 1H), 6.69 (dd, 1H), 6.75 (s, 1H), 6.87 (dd, 1H), 7.07 (t, 1H), 7.22-7.27 (m, 3H), 7.52 (t, 1H), 7.65 (d, 1H), 7.79 (t, 1H) and 8.29 (dd, 1H).

Route 2



2-Ethyl-3,1-[4H]benzoxazin-4-one (7)

Anthranilic acid (100 g, 0.73 mol) and propionic anhydride (420 mL) were heated to 140 °C for 3.5 h, in a flask equipped with Claisen-distillation head. The temperature was increased to 170-180 °C, and propionic acid and the propionic anhydride were removed under reduced pressure to give a brown solid, which was triturated with hexane, filtered and dried to give the *title* benzoxazine (7) (115.8 g, 91%) as an off white solid: δ (360 MHz; CDCl_3) 1.35 (t, 3H), 2.71 (q, 2H), 7.45-7.50 (m, 1H), 7.55 (d, 1H), 7.75-7.79 (m, 1H), 8.16 (dd, 1H).

2-Ethyl-3-(4'-fluoro-phenyl)-quinazolin-4-one (8)

A solution of the benzoxazine (7) (5.0 g, 28.6 mmol) and 4-fluoroaniline (3.37 g, 30.3 mmol) in chloroform (12 mL) was heated to reflux overnight. After complete consumption of the benzoxazine (7) as monitored by TLC, the solvent was removed and the residual off white solid was taken up in ethylene glycol (8 mL) to which sodium hydroxide (51 mg) was added and the suspension was heated to 120-140°C in a distillation apparatus. The water produced was distilled off during a 5 h heating period, and the dark solution allowed to cool to ambient temperature overnight. The pH was adjusted to 7-8 by addition of 3% aqueous hydrochloric acid, and the precipitate was filtered and dried to give the *title* quinazolinone (8) (4.42 g, 58%) as a white solid:

(360 MHz; CDCl₃) 1.23 (t, 3H), 2.44 (q, 2H), 7.24 (apparent d, 4H), 7.44-7.49 (m, 1H), 7.71-7.79 (m, 2H), 8.26 (dd, 1H).

2-(1'-Bromoethyl)-3-(4''fluorophenyl)-quinazolin-4-one (9)

To a stirring mixture of the quinazolinone (8) (94.0 g, 0.35 mol), anhydrous sodium acetate (35.2 g, 0.43 mol) and glacial acetic acid (448 mL) at 40°C under a nitrogen atmosphere, was added dropwise a solution of bromine (76.8 g, 0.48 mol) in glacial acetic acid (243 mL) whilst maintaining the mixture at approximately 40°C over a 5 h period. The mixture was poured onto water (5.26 L), stirred for 1 h and filtered. The filter cake was washed with warm water (2.8 L) and dried to give the *title* bromide (9) (117.2 g, 96%) as a white solid:

δ (360 MHz; CDCl₃) 1.99 (d, 3H), 4.48 (q, 1H), 7.07-7.25 (m, 3H), 7.44-7.52 (m, 2H), 7.73-7.75 (m, 2H), 8.21 (d, 1H).

2-[(1'-Methylamino)-ethyl]-3-(4''-fluorophenyl)-quinazolin-4-one (10)

A 8.03M solution of methylamine in ethanol (923 mL, 7.41 mol), was added to the bromide (9) (117.0 g, 0.34 mol) and the suspension was warmed to 40°C. After 1 h the starting material was fully consumed as monitored by TLC and the solvent and excess methylamine were removed under reduced pressure. The residue was taken up in dichloromethane (1.2 L), stirred for 1 h at ambient temperature, with the precipitated

methylammonium hydrochloride being filtered off, and washed with dichloromethane (117 mL). The combined organic phases were concentrated and dried (MgSO₄) to give the *title* amine (10) quantitatively as a white solid:

δ (360 MHz; CDCl₃) 1.30 (d, 3H), 2.33 (s, 3H), 3.38 (q, 1H), 7.26-7.34 (m, 4H), 7.51-7.55 (m, 1H), 7.76-7.85 (m, 2H), 8.31 (dd, 1H).

2-{1'-[N-(3''-trifluoromethylphenylcarbamoyl)-N-methyl-amino]ethyl}-3-(4'''fluorophenyl)-quinazolin-4-one (11)

To a solution of the amine (10) (18.9 g, 0.065 mol) and chloroform (150 mL) was added a solution of 3-trifluoromethylphenyl isocyanate (11.9 g, 0.065 mol) in chloroform (39 mL) whilst maintaining the temperature below 30°C. The mixture was stirred at ambient temperature for 1 h, and the precipitate formed was filtered, washed with chloroform and dried to give the *title* urea (11) (20.2 g, 65%) as an off white solid:

δ (360 MHz; CDCl₃) 1.55 (d, 3H), 2.92 (s, 3H), 5.16 (q, 1H), 7.09 (br s, 1H), 7.18-7.23 (m, 1H), 7.29-7.30 (m, 3H), 7.34-7.43 (m, 2H), 7.55-7.58 (m, 3H), 7.80-7.88 (m, 2H), 8.31 (d, 1H).

2-{1'-[N-(2''-methoxy-5''-trifluoromethylphenylcarbamoyl)-N-methyl-amino]ethyl}-3-(4'''fluorophenyl)-quinazolin-4-one (12)

A solution of 2-methoxy-5-trifluoromethylaniline (42 mg, 0.22 mmol) in ethyl acetate (1.2 mL) was added to a solution of triphosgene (80 mg, 0.27 mmol) in ethyl acetate (1.2 mL), catalytic amounts of charcoal were added and the mixture heated to reflux for 2 h. After cooling to ambient temperature the solvent was evaporated under reduced pressure, with the resulting residue being taken up in chloroform (1.2 mL). A solution of the amine (10) (65 mg, 0.22 mmol) in chloroform (1.2 mL) was added and the mixture stirred at ambient temperature until the starting material was fully consumed. The solvent was evaporated and the crude product was subjected to silica-gel column chromatography [eluent: Dichloromethane -> Ethyl acetate], with the *title* urea (12) (98 mg, 87%) being isolated as white solid:

δ (360 MHz; CDCl_3) 1.40 (d, 3H), 2.88 (s, 3H), 3.83 (s, 3H), 5.25 (q, 1H), 6.80 (d, 1H), 6.97-7.03 (m, 2H), 7.13-7.18 (m, 3H), 7.20-7.25 (m, 1H), 7.40-7.45 (m, 1H), 7.65-7.74 (m, 2H), 8.18-8.22 (m, 2H).

N-{1-[3-(4-Fluoro-phenyl)-4-oxo-3,4-dihydro-quinazolin-2-yl]-ethyl}-N-methyl-3-trifluoromethyl-benzamide (13)

To a solution of the amine (10) (50 mg, 0.17 mmol) and *N,N*-diisopropylethylamine (0.03 mL, 0.17 mmol) in dichloromethane (0.5 mL) was added 3-trifluoromethylbenzoyl chloride (0.03 mL, 0.19 mmol). The mixture was stirred overnight at ambient temperature under a nitrogen atmosphere. After 12 h a few crystals of 4-dimethylaminopyridine were added, and stirring was continued for another 12 h. The mixture was quenched with water, washed with saturated aqueous sodium hydrogen carbonate, dried (MgSO_4), filtered and concentrated to furnish the *title* benzamide (13) quantitatively as a white foam:

δ (360 MHz; CDCl_3) 1.53 (d, 3H), 3.16 (s, 3H), 5.36 (q, 1H), 7.24-7.36 (m, 3H), 7.45-7.56 (m, 3H), 7.64-7.82 (m, 5H), 8.29 (br d, 1H).

N-{1-[3-(4-Fluoro-phenyl)-4-oxo-3,4-dihydro-quinazolin-2-yl]-ethyl}-N-methyl-3-trifluoromethyl-benzenesulfonamide (14)

To a solution of the amine (10) (50 mg, 0.17 mmol) and *N,N*-diisopropylethylamine (0.03 mL, 0.17 mmol) in dichloromethane (0.5 mL) was added 3-trifluoromethylphenylsulfonyl chloride (0.03 mL, 0.19 mmol), and the mixture was stirred at ambient temperature under a nitrogen atmosphere. After 12 h a few crystals of 4-dimethylaminopyridine were added, and stirring was continued for another 12 h. The mixture was quenched with water, washed with saturated aqueous sodium hydrogen carbonate, dried (MgSO_4), filtered and concentrated to furnish the *title* sulfonamide (14) quantitatively as a white foam:

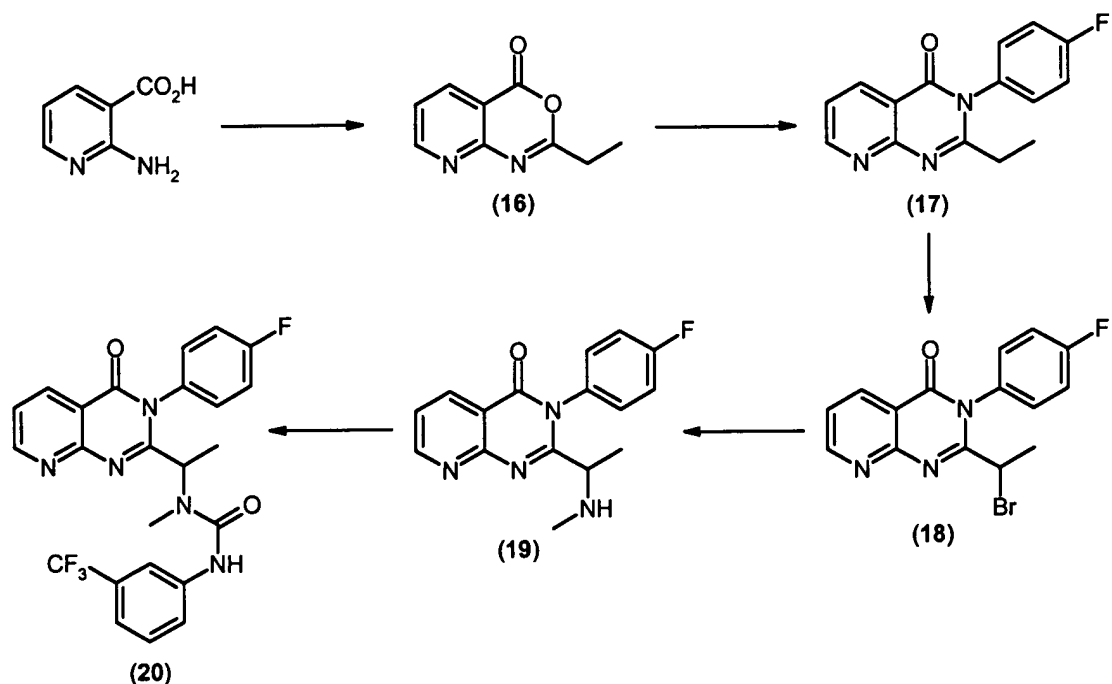
δ (360 MHz; CDCl_3) 1.28 (d, 3H), 3.16 (s, 3H), 4.92 (q, 1H), 7.18-7.30 (m, 2H), 7.35-7.50 (m, 5H), 7.62 (br d, 1H), 7.70 (dt, 1H), 7.79 (br d, 1H), 7.87 (br s, 1H), 8.25 (dd, 1H).

2-{1-[Bis-(2-methoxy-ethyl)-amino]-ethyl}-3-(4-fluoro-phenyl)-3*H*-quinazolin-4-one (15)

A solution of the bromide (9) (71.5 mg, 0.21 mmol), tetrahydrofuran (1.0 mL) and *bis*-(2-methoxyethyl)amine (0.33 mL, 2.26 mmol) was heated to 70°C. After 6.5 h, the reaction was partly concentrated, and ethyl acetate (1 mL) and water (1 mL) were added with the mixture being stirred vigorously for 1 h. The organic layer was separated, washed with water (2 x 1 mL), dried (MgSO₄), filtered and concentrated to give the *title* amine (15) (61 mg, 74%) as an oil:

δ (360 MHz; CDCl₃) 1.35 (d, 3H), 2.51-2.56 (m, 2H), 2.74-2.83 (m, 2H), 2.98-3.11 (m, 4H), 3.12 (s, 6H), 3.79 (q, 1H), 7.13-7.27 (m, 3H), 7.45-7.54 (m, 2H), 7.74-7.76 (m, 2H), 8.26 (d, 1H).

Route 3



2-Ethyl-pyrido[2,3-*d*][1,3]oxazin-4-one (16)

A suspension of 2-aminonicotinic acid (1.0 g 7.24 mmol) in propionic anhydride (10 mL) of was heated to 120°C for 1 h under nitrogen atmosphere. The temperature was

increased to 167°C to remove the propionic acid and propionic anhydride under reduced pressure. The resulting brown solid was triturated with hexane and dried to give the *title* pyrido-oxazine (16) (1.10 g, 86%) as a white solid:

δ (360 MHz; CDCl₃) 1.43 (t, 3H), 2.82 (q, 2H), 7.48 (dd, 1H), 8.53 (dd, 1H), 8.98 (d, 1H).

2-Ethyl-3-(4-fluoro-phenyl)-3*H*-pyrido[2,3-*d*]pyrimidin-4-one (17)

A suspension of the pyrido-oxazine (16) (0.5 g, 2.84 mmol) and 4-fluoroaniline (0.32 g, 2.84 mmol) in toluene (21 mL) was heated to reflux overnight. The solution was concentrated to dryness under reduced pressure and ethylene glycol (2 mL) and sodium hydroxide (5 mg) were added. The mixture was heated again to 120°C for 4 h, and the ethylene glycol was distilled off under reduced pressure. The resulting dark brown solid was subjected to silica-gel column chromatography [eluent: Ethyl acetate:Hexane, 20:80, v/v -> 100:0, v/v] to give the *title* pyrido-pyrimidinone (17) (244 mg, 32%) as a beige solid:

δ (360 MHz; CDCl₃) 1.31 (t, 3H), 2.49 (q, 2H), 7.25-7.28 (m, 4H), 7.44 (dd, 1H), 8.59 (dd, 1H), 9.00 (dd, 1H).

2-(1-Bromo-ethyl)-3-(4-fluoro-phenyl)-3*H*-pyrido[2,3-*d*]pyrimidin-4-one (18)

A suspension of the quinazolinone (17) (50.0 g, 0.56 mmol) and anhydrous sodium acetate (56 mg) in glacial acetic acid (1.0 mL) was heated to 40°C and treated dropwise with a solution of bromine (90 mg, 0.56 mmol) in glacial acetic acid (5 mL). Once the starting material was fully consumed by TLC analysis, the mixture was poured onto water (5 mL), basified with saturated aqueous sodium hydrogen carbonate and extracted with *tert*-butylmethyl ether. The combined organic phases were dried (MgSO₄), filtered, and concentrated to a solid which was purified by silica-gel column chromatography [eluent: Ethyl acetate:Hexane, 50:50, v/v] to give the *title* bromide (18) (166 mg, 85%) as an off white solid:

δ (360 MHz; CDCl₃) 1.21 (d, 3H), 4.58 (q, 1H), 7.15-7.34 (m, 3H), 7.51 (dd, 1H), 7.55-7.60 (m, 1H), 8.62 (dd, 1H), 9.05 (dd, 1H).

3-(4-Fluoro-phenyl)-2-(1-methylamino-ethyl)-3*H*-pyrido[2,3-*d*]pyrimidin-4-one (19)

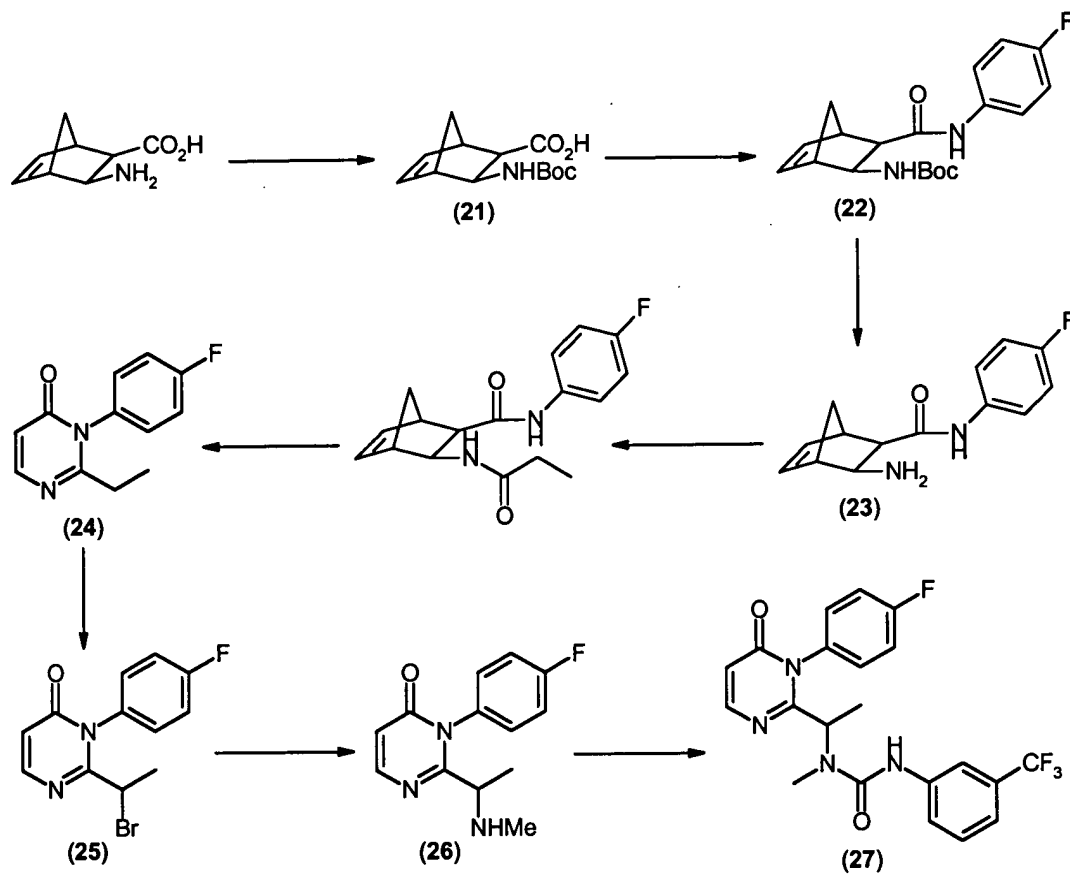
A suspension of the bromide (18) (80 mg, 0.23 mmol) and 8.03M solution of methylamine in ethanol (0.64 mL) was heated to 40°C for 3 h, and stirred at ambient temperature overnight. The resulting suspension was concentrated to dryness under reduced pressure, and the resulting light brown solid was taken up in dichloromethane and filtered through a silica-gel plug to give the *title* amine (19) (57 mg, 83%) as a beige solid (The crude material was directly taken through the next step without further purification).

1-{1-[3[(4-Fluoro-phenyl)-4-oxo-3,4-dihydro-pyrido[2,3-*d*]pyrimidin-2-yl]-ethyl]-1-methyl-3-(3-trifluoromethyl-phenyl)-urea (20)

To a solution of the amine (19) (30 mg, 0.1 mmol) in dichloromethane (0.2 mL) was added 3-trifluoromethylphenyl isocyanate (19 mg, 0.1 mmol). The mixture was stirred at ambient temperature overnight, after which, the precipitate was filtered, washed with dichloromethane and dried to give the *title* urea (20) (6 mg, 13%) as a white solid:

δ (360 MHz; CDCl₃) 2.17 (d, 3H), 3.15 (s, 3H), 5.23 (q, 1H), 7.15 (br s, 1H), 7.22-7.31 (m, 4H), 7.38 (t, 1H), 7.49 (d, 1H), 7.50-7.56 (m, 2H), 7.62 (br s, 1H), 8.62 (dd, 1H), 9.00 (d, 1H).

Route 4



3-Exo-tert-butoxycarbonylamino-bicyclo[2.2.1]hept-5-ene-2-exo-carboxylic acid (21)

A solution of 3-exo-amino-bicyclo[2.2.1]hept-5-ene-2-exo-carboxylic acid (1.0 g, 6.5 mmol), 1N aqueous sodium hydroxide (6.5 mL) and dioxane (6.5 mL) was cooled on an ice-bath, and di-*tert*-butyl dicarbonate (3.0 g, 13.8 mmol) was added with stirring continuing for 10 minutes at 0°C and 6 h at ambient temperature. The solvent was partially evaporated and the pH was adjusted to 1-2 by addition of 1N aqueous potassium hydrogen sulfate (~8 mL). The aqueous phase was extracted with ethyl acetate (3 x 10 mL), and the combined organic phases were dried (MgSO₄), and concentrated to give the *title acid* (21) (1.41 g, 85%) as a white solid:

δ (360 MHz; CDCl₃) 1.70 (s, 9H), 1.89 (m, 1H), 2.31 (m, 1H), 2.84 (m, 1H), 2.98 (m, 1H), 3.22 (m, 1H), 4.20 (m, 1H), 6.44 (m, 2H), 7.21 (m, 1H).

[3-Exo-(4-fluoro-phenylcarbamoyl)-bicyclo[2.2.1]hept-5-en-2-exo-yl]-carbamic acid tert-butyl ester (22)

A solution of the acid (21) (1.4 g, 5.5 mmol) in anhydrous tetrahydrofuran (20 mL) was cooled to -10°C and treated dropwise with triethylamine (0.77 mL, 5.5 mmol), followed by isobutyl chloroformate (0.72 mL, 5.5 mmol). The resulting suspension was stirred for 10 minutes at -10°C before a solution of 4-fluoroaniline (0.53 mL, 5.5 mmol) in anhydrous tetrahydrofuran (5 mL) was added, with the temperature being kept between -8 and -17°C for 5 h and mixture allowed to warm to ambient temperature overnight. The triethylaminehydrochloride was filtered off, washed with tetrahydrofuran, and the solvent was evaporated to dryness, with the resulting solids being washed with water (20 mL) and dried to give the title carbamic acid ester (22) quantitatively as a white solid:

δ (360 MHz; CDCl_3) 1.24 (s, 9H), 1.66 (d, 1H), 2.06 (d, 1H), 2.45 (d, 1H), 2.71 (br s, 1H), 3.08 (br s, 1H), 3.93-4.06 (m, 1H), 4.96 (d, 1H), 6.18-6.24 (m, 2H), 6.94-7.02 (m, 2H), 7.48-7.50 (m, 2H), 7.98 (br s, 1H).

3-Exo-amino-bicyclo[2.2.1]hept-5-ene-2-exo-carboxylic acid (4-fluoro-phenyl)-amide (23)

To a solution of the carbamic acid ester (22) (2.24 g) in dichloromethane (50 mL) was added trifluoroacetic acid (5 mL). The solution was stirred at ambient temperature for 4 h before water (50 mL) was added. The aqueous phase was separated and washed with dichloromethane (25 mL), filtered and basified with 2N sodium hydroxide. The basified aqueous phase was extracted with dichloromethane (2 x 25 mL), and the combined organic phases were washed with brine (10 mL), dried (MgSO_4), and concentrated to give the title amine (23) (870 mg, (64% over 2 steps from (21)) as a solid: δ (360 MHz; CDCl_3) 1.59 (d, 1H), 1.71 (br d, 2H), 2.13 (d, 1H), 2.37 (d, 1H), 2.59 (br s, 1H), 3.09 (br s, 1H), 3.24 (d, 1H), 6.20 (br s, 2H), 6.99 (t, 2H), 7.47-7.51 (m, 2H), 8.54 (br s, 1H).

2-Ethyl-3-(4-fluoro-phenyl)-3H-pyrimidin-4-one (24)

A solution of the amine (23) (43 mg, 0.17 mmol) in triethylorthopropionate (0.5 mL, 2.38 mmol) was heated to 100°C for 26 h, after which the excess solvent and volatile

co-products were evaporated under reduced pressure to give the *title* pyrimidinone (**24**) (41 mg, quant.) as a crystalline solid:

δ (360 MHz; CDCl₃) 1.17 (t, 3H), 2.37 (q, 2H), 6.45 (d, 1H), 7.17-7.27 (m, 4H), 7.93 (d, 1H).

2-(1-Bromo-ethyl)-3-(4-fluoro-phenyl)-3H-pyrimidin-4-one (25)

A solution of the pyrimidinone (**24**) (150 mg, 0.69 mmol) and sodium acetate (140 mg) in glacial acetic acid (1.5 mL) was warmed to 40°C and treated dropwise with a preformed bromine solution (0.7 mL bromine in 10 mL of glacial acetic acid) (0.55 mL, 0.69 mmol). After 2 h, water (20 mL) was added to the mixture which was subsequently basified with potassium carbonate, and extracted with dichloromethane (2 x 10 mL). The combined organic phases were washed with water (10 mL), dried (MgSO₄), and concentrated to give the *title* bromide (**25**) (196 mg, 96%) as a pale brown oil:

δ (360 MHz; CDCl₃) 1.95 (d, 3H), 4.46 (q, 1H), 6.50 (d, 1H), 7.12-7.16 (m, 1H), 7.20-7.28 (m, 2H), 7.43-7.50 (m, 1H), 8.81 (d, 1H).

3-(4-Fluoro-phenyl)-2-(1-methylamino-ethyl)-3H-pyrimidin-4-one (26)

The bromide (**25**) (190 mg, 0.64 mmol) was dissolved in a 33% solution of methylamine in ethanol (5 mL) and stirred at ambient temperature. After 3.5 h, the solvent and excess methylamine were evaporated under reduced pressure and residue was partitioned between water (10 mL) and dichloromethane (10 mL). The organic layer was washed again with water (10 mL), dried (MgSO₄), and concentrated to give the *title* amine (**26**) (120 mg, 76%) as a yellow oil:

δ (360 MHz; CDCl₃) 1.20 (d, 3H), 2.23 (s, 3H), 3.26 (q, 1H), 6.44 (d, 1H), 7.16-7.33 (m, 4H), 7.98 (d, 1H).

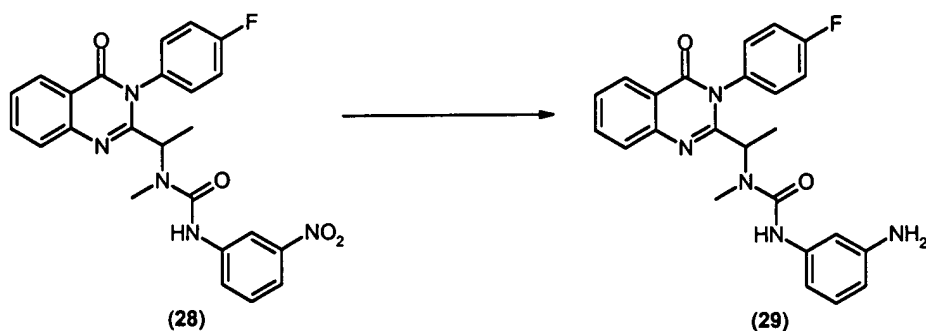
1-{1-[1-(4-Fluoro-phenyl)-6-oxo-1,6-dihydro-pyrimidin-2-yl]-ethyl}-1-methyl-3-(3-trifluoromethyl-phenyl)-urea (27)

The amine (**26**) (12 mg, 0.05 mmol) and 3-trifluoromethylphenyl isocyanate (7.5 L, 0.05 mmol) were dissolved in chloroform (0.2 mL) and stirred at ambient temperature. After complete conversion of the starting material by TLC analysis, the

solvent was removed and the resulting residue was subjected to silica-gel column chromatography [eluent: Dichloromethane] to give the *title* urea (27) (14 mg, quant.) as a solid:

δ (360 MHz; CDCl_3) 1.61 (m, 3H), 2.87 (s, 3H), 5.04 (m, 1H), 6.41 (m, 1H), 6.64 (s, 1H), 7.11-7.48 (m, 8H), 7.86 (m, 1H).

Nitro Reduction to Anilines

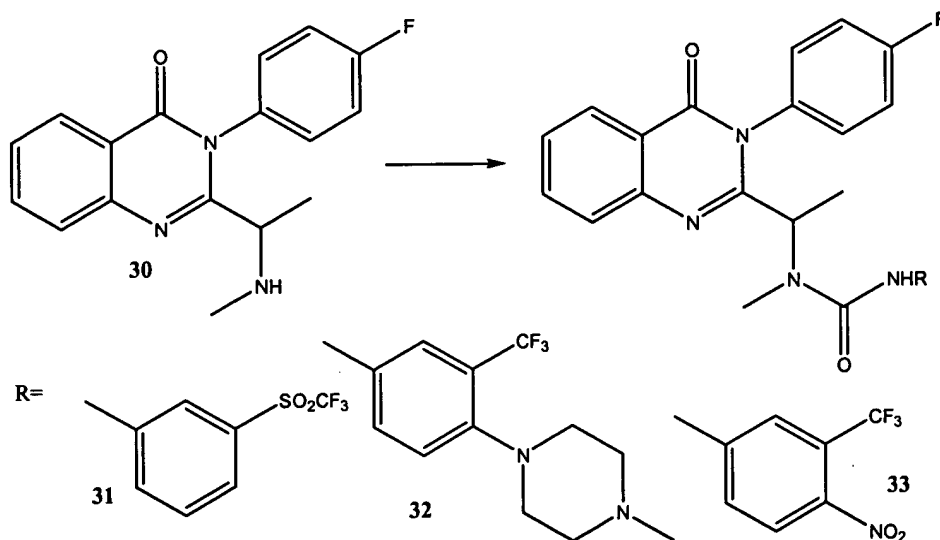


2-{1'-[N-(3''-amino-carbamoyl)-N-methyl-amino]ethyl}-3-(4'''fluorophenyl)-quinazolin-4-one (29)

A solution of 2-{1'-[N-(3''-nitro-carbamoyl)-N-methyl-amino]ethyl}-3-(4'''fluorophenyl)-quinazolin-4-one (28) (168 mg, 0.036 mmol), dichloromethane (5 mL) and ethyl acetate (5 mL) was stirred under a hydrogen atmosphere in the presence of 10% activated palladium on charcoal until the starting material was fully consumed. The mixture was filtered through celite and concentrated to dryness to give the *title* aniline (29) (100 mg, 65%):

δ (360 MHz; CDCl_3) 1.48 (d, 3H), 2.88 (s, 3H), 5.17 (q, 1H), 6.39 (br s, 1H), 6.50 (br s, 1H), 6.92 (br s, 1H), 6.60 (br s, 1H), 7.02 (t, 1H), 7.15-7.33 (m, 4H), 7.49-7.55 (m, 1H), 7.75-7.82 (m, 2H), 8.27 (d, 1H).

An *in situ* method for derivatizing the pendant amine was developed, as exemplified by the following protocol:



1-{1-[3-(4-Fluoro-phenyl)-4-oxo-3,4-dihydro-quinazolin-2-yl]-ethyl}-1-methyl-3-(3-trifluoromethanesulfonyl-phenyl)-urea (**31**)

A solution of triphosgene (160 mg, 0.54 mmol) and ethyl acetate (2.5 mL) was added to a solution of (3-aminophenyl)trifluoromethyl sulfone (100 mg, 0.44 mmol) and ethyl acetate (2.5 mL). After stirring for 5 min the mixture was refluxed until it went clear. The mixture was concentrated and redissolved in chloroform (2.5 mL) to which a solution of the amine (**30**) (130 mg, 0.44 mmol) in chloroform (2.5 mL) was added. Once all of the starting material had been consumed by TLC, the solvent was removed in *vacuo* and subjected to silica gel column chromatography using dichloromethane then ethyl acetate as eluent to give the *title urea* (**31**) (226 mg, 94%) as a solid.

δ_H (360 MHz; $CDCl_3$) 1.43 (d, 3H), 2.88 (s, 3H), 4.98 (br s, 1H), 7.08-7.14 (m, 1H), 7.16-7.31 (m, 3H), 7.45-7.54 (m, 3H), 7.60 (d, 1H), 7.69-7.80 (m, 3H), 7.96 (d, 1H) and 8.22 (d, 1H).

1-{1-[3-(4-Fluoro-phenyl)-4-oxo-3,4-dihydro-quinazolin-2-yl]-ethyl}-3-(3-trifluoromethyl-4-nitro-phenyl)-1-methyl-urea (**32**)

A solution of triphosgene (80 mg, 0.27 mmol) and ethyl acetate (1.2 mL) was added to a solution of 4-nitro-3-trifluoromethylaniline (45 mg, 0.22 mmol) and ethyl acetate (1.2 mL). After stirring for 5 min the mixture was refluxed until it went clear. The mixture was concentrated and redissolved in chloroform (1.2 mL) to which a solution of the

amine (30) (65 mg, 0.22 mmol) in chloroform (1.2 mL) was added. Once all of the starting material had been consumed by TLC, the solvent was removed in *vacuo* and subjected to silica gel column chromatography using dichloromethane then ethyl acetate as eluent to give the *title urea* (32) (112 mg, 97%) as a solid.

δ_H (360 MHz; $CDCl_3$) 1.45 (d, 3H), 2.80 (s, 3H), 4.90 (br s, 1H), 7.05-7.25 (m, 4H), 7.48 (t, 1H), 7.63-7.79 (m, 4H), 7.86 (d, 1H) and 8.19 (d, 1H).

1-{1-[3-(4-Fluoro-phenyl)-4-oxo-3,4-dihydro-quinazolin-2-yl]-ethyl}-3-[3-trifluoromethyl-4-(4-methyl)-piperazin-1-yl]-phenyl]-1-methyl-urea (33)

A solution of triphosgene (80 mg, 0.27 mmol) and ethyl acetate (1.2 mL) was added to a solution of 4-(*N*-methylpiperazine)-3-trifluoromethyl aniline (56 mg, 0.22 mmol) and ethyl acetate (1.2 mL). After stirring for 5 min the mixture was refluxed until it went clear. The mixture was concentrated and redissolved in chloroform (1.2 mL) to which a solution of the amine (30) (65 mg, 0.22 mmol) in chloroform (1.2 mL) was added. Once all of the starting material had been consumed by TLC, the solvent was removed in *vacuo* and subjected to silica gel column chromatography using ethyl acetate then 5-10% methanol in dichloromethane as eluent to give the *title urea* (33) (25 mg, 20%) as a solid.

δ_H (360 MHz; $CDCl_3$) 1.38 (d, 3H), 1.97 (br s, 2H), 2.26 (s, 3H), 2.47 (br s, 2H), 2.72-2.86 (m, 7H), 4.92-5.05 (m, 1H), 6.80-6.93 (br s, 1H), 7.08 (t, 1H), 7.12-7.27 (m, 4H), 7.34 (s, 1H), 7.41 (t, 2H), 7.63-7.74 (m, 2H) and 8.16 (d, 1H).

All of the references cited above are hereby incorporated by reference herein.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.